The Orphan Response Regulator DigR Is Required for Synthesis of Extracellular Matrix Fibrils in *Myxococcus xanthus*†

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In *Myxococcus xanthus*, two-component systems have crucial roles in regulating motility behavior and development. Here we describe an orphan response regulator, consisting of an N-terminal receiver domain and a C-terminal DNA binding domain, which is required for A and type IV pilus-dependent gliding motility. Genetic evidence suggests that phosphorylation of the conserved, phosphorylatable aspartate residue in the receiver domain is required for DigR activity. Consistent with the defect in type IV pilus-dependent motility, a *digR* mutant is slightly reduced in type IV pilus biosynthesis, and the composition of the extracellular matrix fibrils is abnormal, with an increased content of polysaccharides and decreased accumulation of the FibA metalloprotease. By using genome-wide transcriptional profiling, 118 genes were identified that are directly or indirectly regulated by DigR. These 118 genes include only 2, *agmQ* and *cheY4*, previously implicated in A and type IV pilus-dependent motility, respectively. In silico analyses showed that 36% of the differentially expressed genes are likely to encode exported proteins. Moreover, four genes encoding homologs of extracytoplasmic function (ECF) sigma factors, which typically control aspects of cell envelope homeostasis, are differentially expressed in a *digR* mutant. We suggest that the DigR response regulator has an important function in cell envelope homeostasis and that the motility defects in a *digR* mutant are instigated by the abnormal cell envelope and abnormal expression of *agmQ* and *cheY4*.

*Myxococcus xanthus* cells construct two morphologically distinct biofilms depending on their nutritional status (46). In the presence of nutrients, colonies are formed in which cells at the edge spread outwards in a coordinated manner. In the absence of nutrients, cells aggregate to construct multicellular, spore-filled fruiting bodies. Formation of both types of biofilms depends crucially on the ability of cells to display active movement and to regulate their motility behavior appropriately.

*M. xanthus* cells move by gliding and possess two different motility systems, referred to as the S and A motility systems (22). S motility, which is the *M. xanthus* equivalent of the twitching motility of *Neisseria* and *Pseudomonas* species, depends on three major extracellular components, type IV pili (Tfp) (25, 58), extracellular matrix fibrils (3, 42), and lipopolysaccharide (LPS) O antigen (11), and is generally functional only when cells are within contact distance of each other (22). Tfp are unipolar structures that extend more than a cell length from the pole of a cell (25). Motive force is generated by retraction of Tfp (32, 45, 50), which depends on contact with the polysaccharide portion of extracellular matrix fibrils on a neighboring cell (30). Extracellular matrix fibrils are composed of equal amounts of polysaccharide and proteins and coat the cell surface, forming a network in which neighboring cells are interconnected (6). These fibrils are filamentous structures 10 to 30 nm in diameter emanating from all over the cell (3, 7). Recently, it has been proposed that these fibrils more likely form a matrix of carbohydrates and protein surrounding the body of a cell (30). The function of the LPS O antigen in S motility is currently unknown. The A motility system in *M. xanthus* is operational in isolated cells (22). A motility depends on a poorly defined mechanism that has been suggested to generate motive force by means of slime extrusion from polarly localized nozzle-like structures (57). Generally, the two motility systems are functionally independent, and mutants deficient in one system retain the ability to move by means of the remaining system (22). Mutants with mutations in both systems are nonmotile (22). However, three proteins affecting both systems have been identified—the transcriptional regulator Nla24 (29), the MglA protein, which is a member of the Ras/Rab/Rho superfamily of GTPases (18), and the MglB protein, which acts to stabilize MglA (17)—thus suggesting that the two motility systems may share functional components.

Two-component signal transduction systems are involved in a wide variety of responses to environmental cues in bacteria (49) and also have decisive roles in controlling complex processes such as the cell cycle (5) and initiation of sporulation (20). Gliding motility as well as fruiting body formation in *M. xanthus* also involves a large number of two-component signal transduction proteins (26). Classical two-component systems comprise a sensor histidine protein kinase and a response regulator protein. Typically, sensor kinases are multidomain proteins and contain a nonconserved sensory input domain, which is responsible for detecting a particular stimulus or ligand, and a conserved kinase domain. Likewise, response regulators are often multidomain proteins with a conserved receiver domain and a variable output domain. The kinase autophosphorylates on a conserved histidine residue. The phosphoryl group is subsequently transferred to a conserved
aspartate residue in the receiver domain of the cognate response regulator, resulting in activation of the output domain. Generally, adjacent genes encode the proteins in a two-component system (49). However, many of the two-component systems involved in fruiting body formation in M. xanthus are encoded by orphan genes, i.e., a kinase gene is not flanked by a gene encoding a response regulator, and vice versa. Interestingly, approximately 50% of the 164 histidine protein kinases and approximately 40% of the 130 response regulators encoded on the M. xanthus genome are orphans (Xingqi Shi, personal communication).

To expand our understanding of the design of two-component signaling pathways involved in regulating motility and fruiting body formation in M. xanthus, we have initiated a systematic analysis of the function of orphan two-component signal transduction proteins in M. xanthus. Here we report the characterization of an orphan response regulator, DigR, which is composed of an N-terminal receiver domain and a C-terminal DNA binding domain. We report that digR is required for A and Tfp-dependent motility as well as fruiting body formation, and we provide data suggesting that DigR is required for normal synthesis of the extracellular matrix fibrils.

Moreover, we present genetic evidence suggesting that DigR is activated by phosphorylation. Using genome-wide transcriptional profiling, we identify 118 genes that are directly or indirectly regulated by DigR. Thirty-six percent of the genes encode proteins likely to be exported. Moreover, 4 of the 118 genes encode homologs of extracytoplasmic function (ECF) sigma factors. On the basis of these findings, we suggest that DigR may play an important role in cell envelope homeostasis.

MATERIALS AND METHODS

**Bacterial strains, growth, development, motility, and agglutination assay.** Escherichia coli Top10 (F’ lacZAM15 deoR recA1 araD139 lac proph7697 galU galK rpsL endA1 nupG) (Invitrogen) was used for plasmid constructions. Myxobacterial strains and plasmids are listed in Table 1. E. coli strains were grown at 37°C in Luria-Bertani (LB) broth or on LB plates in the presence of relevant antibiotics. M. xanthus strains were grown at 32°C in 1% CTT broth (21) or on CTT medium supplemented with 1.5% agar. Gentamicin (10 μg/ml), kanamycin (40 μg/ml), or oxytetracycline (10 μg/ml) was added when appropriate. M. xanthus development was assayed on CFF agar (44) as described elsewhere (47). For motility assays, cells were grown in CTT broth to a density of 7 × 10⁶/ml harvested, and resuspended in 1% CTT to a calculated density of 7.0 × 10⁶ cells/ml. Aliquots (5 μl) of cells were placed on 0.5% and 1.5% agar supplemented with 0.5% CTT and incubated at 32°C. After 24 h, colony morphology and colony edges were observed visually with a stereomicroscope (Leica MZ5) and photographed using a Sony 3CCD color video camera. Agglutination assays were carried out as described elsewhere (42).

**Strain construction.** For SA1801, an internal digR fragment was amplified by PCR using primers M60f and M60r, and the PCR product was digested with HindIII and cloned into pBG18 (48) to create pMO70, which was electroporated into DK1622 as described elsewhere (27). For SA1804, a 1-kb fragment of DNA upstream of the digR start codon was amplified by PCR using primers odigR-16f and odigR-4r (Invitrogen) and cloned into pBGS18 (48) to create pMO102. All fragments generated by PCR were sequenced to ensure the absence of mutations. The sequences of primers used for construction are available from the authors upon request.

**Plasmid constructions.** Plasmid pMO80 was generated by digesting chromosomal DNA from SA1801 with XmnI, followed by religation. pMO80 contains pmO70 as well as 7 kb upstream and 3 kb downstream, respectively, from the insertion site. Wild-type digR was cloned in two steps. First, a 4-kb PstI-SmaI fragment containing the upstream region of digR was cloned from pMO80 into pBG18 to produce pMO84. Next, a SmaI-EcoRI fragment containing the digR gene including 500 bp downstream of the stop codon was PCR amplified and cloned into pMO64 to produce pMO85. pMO86 was generated by cloning a HindIII-EcoRI fragment from pMO85 containing the wild-type digR gene into pSWU30. For pMO99, 1-kb DNA fragments upstream and downstream of MXAN5591 were amplified by PCR and assembled in PB1131 in two steps. First, a fragment was generated using primers odigR-15f and odigR-15r. The resulting PCR fragment was digested with HindIII and XbaI and cloned into pMO96. This construct was moved into pSWU30 to yield pMO99. The asparagine residue D53 in DigR was replaced by an asparagine by changing the asparagine codon ACC to ACA by site-directed mutagenesis. A two-step strategy using the mutagenic primer odigR-8rDN was applied to generate a 1.1-kb PCR fragment containing the digR gene including 500 bp downstream of the stop codon. This fragment was cloned into pMO99 to create pMO102. All fragments generated by PCR were sequenced to ensure the absence of mutations. The sequences of primers used for plasmid constructions are available from the authors upon request.

**Plasmid constructions.** Plasmid constructions are available from the authors upon request. For analyses of total PilA and FibA levels, the immunoblots were probed either with anti-PilA antibodies or with anti-FibA monoclonal antibodies (MAb) 2105 (28). The secondary antibodies used were either horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (for PilA) or horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (for FibA). O antigen was quantified in slot blots of whole cells as described previously using MAb 783 (15). Blots were developed using the Renaissance Plus chemiluminescence reagent (NEN Life Sciences).

**Isolation of total RNA and quantitative real-time PCR.** Total RNA was prepared from mid-exponential-phase liquid cultures of M. xanthus. Cells were transferred to a centrifuge tube on ice containing RNase free stop solution (1/10 volume of 5% phenol in 96% ethanol). RNA was extracted by the hot-phenol method (39). Gene expression data obtained by microarray analysis were verified using quantitative real-time PCR as described previously for M. xanthus (38). The sequences of primers used for quantitative real-time PCR are available from the authors upon request.

**AN ORPHAN RESPONSE REGULATOR IN M. XANTHUS**

<table>
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<tr>
<th>Strain</th>
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TABLE 1. Bacterial strains used in this study

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DNA microarray design. A DNA microarray covering 80% of the *M. xanthus* genes was constructed by the *Myxococcus* Microarray Consortium and will be described in detail elsewhere (J. Jakobsen et al., unpublished data). Briefly, primers for the annotated open reading frames (ORFs) of the *M. xanthus* genome were designed using Microarray Architect (23) and used to amplify PCR products of 275 to 325 bp. After gel purification and resuspension in 3× SSC buffer (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (39), PCR products were spotted onto glass slides that had previously been coated with a poly-L-lysine solution (0.0086% poly-L-lysine [Sigma]-0.1% phosphate-buffered saline).

Spotted arrays were processed before use as described elsewhere (12) with the following modifications. Slides were rehydrated in bovine serum albumin (Sigma). Subsequently, slides were wash five times, for 1 min each time, in 2× SSC–0.1% SDS, for 5 min in 0.2× SSC, followed by a short rinse in H2O for 2 s. Subsequently, slides were snap-dried on a heating block at 140°C for 1 to 2 s, followed by UV cross-linking in a Stratalinker. To block free amine, slides were submerged for 15 min in a solution prepared by dissolving 5.5 g succinic anhydride in 335 ml 1-methyl-2-pyrrolidinone and adding 15 ml 1 M NaH2BO3 (pH 8.0). Finally, the PCR products were denatured by placing slides in 95°C water for 1 min. Slides were washed in 95% ethanol, dried by centrifugation, and stored until further use. Prior to use (<48 h), slides were prehybridized at 42°C for 45 min in 4× SSC–0.5% sodium dodecyl sulfate (SDS)–1% bovine serum albumin (Sigma). Subsequently, slides were washed five times, for 1 min each time, in filtered, deionized H2O at room temperature and were dried by centrifugation.

cDNA synthesis, fluorescent labeling, and hybridization. Synthesis of Cy3- or Cy5-labeled cDNA probes was carried out using 25 μg of DNA-free total RNA and a Cy3cribe postlabeling kit (Amersham Biosciences) according to the protocol provided by the manufacturer. Probes were mixed with final concentrations of 3× SSC–0.5% sodium dodecyl sulfate (SDS)–0.1% bovine serum albumin (Sigma). Subsequently, slides were washed five times, for 1 min each time, in filtered, deionized H2O at room temperature and were dried by centrifugation.

Data acquisition and analysis. Microarrays were scanned at two wavelengths (Cy3, 532 nm; Cy5, 632 nm) using a GMS418 microarray scanner (Affymetrix/Genetic Microsystems). Image analysis and processing were performed using the GenePix, version 4.0, software package (Axon Instruments). The ratio-normalized data set (mean ratio of medians = 1) containing the median signal intensity and median signal background from each channel was further analyzed using Acuity, version 4.0 (Axon Instruments), and the Significance Analysis of Microarrays software, version 2.0 (SAM v.2.0), which assigns a score to each feature on a microarray on the basis of changes in gene expression relative to the standard deviation of repeated measurements (52). A filtered subset of all features printed on the array was selected based on the following criteria: the feature was found by the GenePix 4.0 spot-finding algorithm (Fvalue ≥ 0), and the local-background-subtracted median intensity value of either the Cy3 (532-nm) or the Cy5 (635-nm) channel was greater than 500. For statistical significance analysis of the filtered data points, we used SAM v.2.0 to calculate a t-like statistic (d) based on the estimated variance of the data. The delta value of the SAM analysis was chosen as the value where the false-discovery rate became 0%. From this data set, we selected those features that had data points present in 95% of all microarrays, and the ratios were averaged. Finally, this data set was subjected to a threefold cutoff criterion. The resulting set of 118 genes was analyzed for homology and putative function of encoded proteins using BLASTP and SMART (2, 40).

Nucleotide sequence accession number. The sequence reported in this paper has been deposited in the GenBank database (accession no. DQ272385).

RESULTS

Identification of the novel orphan response regulator gene digR. To identify genes in the *M. xanthus* genome (available at http://www.tigr.org) encoding orphan response regulators, we initially identified genes encoding proteins containing a receiver domain. From this list, hybrid kinases were removed. Next, we identified response regulator genes not flanked by a gene encoding a histidine protein kinase. One of the loci identified is shown in Fig. 1. This locus contains an ORF, MXAN5592, encoding a response regulator protein with an N-terminal receiver domain and a C-terminal DNA binding domain. None of the flanking ORFs encode a histidine protein kinase. The gene encoding the orphan response regulator was named digR (defective in gliding regulator).

**digR** spans a region of 594 bp and has a high GC content in the third positions of codons, a feature typical of GC-rich organisms (9, 43). A putative ribosome-binding site (AGGAGA) is located 4 bp upstream from the GTG start codon. The N-terminal part of DigR (amino acids 1 to 118) shares homology with receiver domains (Fig. 2A) and contains the highly conserved signature residues indicative of phosphorylation (Fig. 2A) (49). The C-terminal part of DigR (amino acids 138 to 197) contains a helix-turn-helix (HTH) domain (Fig. 2B) referred to as HTH_1 and HTH_2, respectively, in the SMART and Pfam databases. This domain is found in a large number of DNA binding proteins, including the bacteriophage 434 transcriptional regulators CI and Cro. As shown in Fig. 2B, the conserved residues important for maintaining the hydrophobic core of the HTH motif in the bacteriophage 434 regulators are conserved in the C-terminal domain of DigR (34), supporting the notion that DigR is a DNA binding protein.

**digR** is required for gliding motility and development. To analyze the function of digR, a strain carrying an insertion mutation in the *digR* gene was constructed. Briefly, a 477-bp DNA fragment internal to *digR* was amplified by PCR and cloned into plasmid pBG518. The resulting plasmid, pMO70, was introduced into the fully motile strain DK1622, which served as the wild-type strain in this study. This plasmid is unable to replicate in *M. xanthus* and integrates by a single homologous recombination event that generates a strain containing two truncated copies of the *digR* gene. The resulting strain was designated SA1801 and subjected to further analyses.

To test whether cells containing the digR::pMO70 allele...
were deficient in gliding motility, colony spreading was examined on 1.5% agar plates (Fig. 3 and 4). Under these conditions, DK1622 wild-type cells form large spreading colonies with rafts of cells as well as single cells present at the edge of the advancing colony. In contrast, the digR::pMO70 mutant cells formed a colony with a sharp edge, and only a few rafts and single cells were observed at the edge. The colony formed by SA1801 was light brownish, whereas the characteristic color of a wild-type colony is yellow.

To examine whether digR is important for fruiting body formation, the digR::pMO70 mutant cells were exposed to starvation on CF starvation medium (Fig. 3). At 120 h, wild-type cells had completed fruiting body formation. Even after 120 h of starvation, the digR::pMO70 mutant cells had not formed fruiting bodies. Moreover, the sporulation frequency of the digR::pMO70 cells was less than 0.01% that of wild-type cells.

To determine whether the defects caused by the digR::pMO70 insertion were due to inactivation of the digR gene, we carried out genetic complementation experiments. digR may be cotranscribed with the upstream gene MXAN5591. Consequently, the complementation experiments were carried out with two different digR-containing fragments cloned into pSWU30, which integrates by site-specific recombination at the Mx8 phage attachment site, attB, on the chromosome. Plasmid pMO86 contains a 4,826-bp fragment that includes digR and the two ORFs upstream of digR (Fig. 1). Introduction of starvation, the digR::pMO70 mutant cells had not formed fruiting bodies. Moreover, the sporulation frequency of the digR::pMO70 cells was less than 0.01% that of wild-type cells. To determine whether the defects caused by the digR::pMO70 insertion were due to inactivation of the digR gene, we carried out genetic complementation experiments. digR may be cotranscribed with the upstream gene MXAN5591. Consequently, the complementation experiments were carried out with two different digR-containing fragments cloned into pSWU30, which integrates by site-specific recombination at the Mx8 phage attachment site, attB, on the chromosome. Plasmid pMO86 contains a 4,826-bp fragment that includes digR and the two ORFs upstream of digR (Fig. 1). Introduction
of pMO86 into a strain containing digR::pMO70 gave rise to strain SA1806. As shown in Fig. 3, SA1806 displayed normal motility and development. Introduction of plasmid pMO99, which contains digR, part of MXAN5590, and an in-frame deletion of MXAN5591 (Fig. 1), into the strain containing digR::pMO70 gave rise to strain SA1807. As shown in Fig. 3, SA1807 displayed normal motility and development. Introduction of pSWU30 into the strain containing the digR::pMO70 mutation—giving rise to strain SA1805—rescued neither the motility nor the developmental defects (Fig. 3). Finally, we constructed a strain, SA1804, that carries a markerless deletion mutation in digR. SA1804 contains a 600-bp deletion in digR from position −21 to +580 (Fig. 1). The phenotype of this mutant was identical to that of the insertion mutant SA1801 (Fig. 3), and gliding motility and development were restored by complementation with plasmids pMO86 and pMO99 (data not shown). From these analyses, we conclude that digR is important for gliding motility in vegetative cells and for fruiting body formation in starving cells.

**D53 in DigR is important for function.** Response regulators depend on phosphorylation of the conserved aspartate residue in the receiver domain. To test genetically whether DigR depends on phosphorylation of this aspartate residue, D53, the putative site of phosphorylation in DigR (Fig. 2A), was replaced with an asparagine residue by site-directed mutagenesis. Plasmid pMO102 is similar to pMO99 except that it contains the mutant digR(D53N) allele (Fig. 1). Integration of pMO102 at the attB site in the digR mutant strains SA1801 (which contains the digR::pMO70 allele) and SA1804 (which contains the ΔdigR allele) resulted neither in complementation of the motility defects nor in complementation of the fruiting body formation defect (Fig. 3 and data not shown). From these results, we conclude that D53 in DigR is necessary for function. In addition, these data provide genetic evidence that DigR depends on phosphorylation for full activity.

**DigR is required for normal A and S motility.** To analyze which of the two gliding motility systems is deficient in a digR mutant, the digR::pMO70 insertion mutation was introduced by Mx4-dependent generalized transduction into DK1218, which is deficient in A motility and proficient in S motility (A−S+), giving rise to strain SA1803, and into DK1300, which is proficient in A motility and deficient in S motility (A+S−), giving rise to strain SA1802. Subsequently, the motility characteristics of these two strains were determined on a soft agar surface (0.5% agar) and on a hard agar surface (1.5% agar) and compared to those of A−S+, A−S−, A+S+, and A+S− reference strains (Fig. 4). Soft agar surfaces favor social gliding, whereas hard agar surfaces favor adventitious gliding (41).

On hard agar, wild-type cells form large colonies with rafts as well as single cells at the edge (Fig. 4A). A−S+ colonies have a few rafts at the edge. In contrast, A−S− cells are nonmotile and form colonies with a sharp edge. The digR mutant cells form colonies with only a few rafts and single cells at the edge. Introduction of the digR mutation into A−S− cells results in the formation of sharp-edged colonies. Introduction of the digR mutation into A−S− cells results in the formation of colonies with only a few single cells present at the edge. On soft agar (Fig. 4B), wild-type and A−S+ cells form large colonies, A−S− cells form small colonies, and A−S− cells form nonsprawling colonies. The digR mutation gave rise to the formation of nonsprawling colonies both in the wild-type background (SA1801) and in the A−S+ (SA1803) and A−S− (SA1802) backgrounds. Consistently, examination of the behavior of digR mutant cells using time-lapse microscopy showed that single cells as well as groups of cells behaved similarly and displayed little movement. From these analyses we conclude that DigR is absolutely required for S motility and partially required for A motility.

**Cell surface structures in a digR mutant.** Next, we examined whether DigR is required for synthesis of any of the known structures required for S motility (Tfp, LPS O antigen, and extracellular matrix fibrils). We determined the total accumulation of the Tfp subunit protein PilA by immunoblot analyses of whole-cell lysates using anti-PilA antibodies, with the pilA::Tc− strain, DK10407, as a negative control. As shown in Fig. 5A, the total level of PilA in the strain containing the digR::pMO70 insertion mutation was introduced by Mx4-dependent generalized transduction into DK1218, which is deficient in A motility and proficient in S motility (A−S+), giving rise to strain SA1803, and into DK1300, which is proficient in A motility and deficient in S motility (A+S−), giving rise to strain SA1802. Subsequently, the motility characteristics of these two strains were determined on a soft agar surface (0.5% agar) and on a hard agar surface (1.5% agar) and compared to those of A−S+, A−S−, A+S+, and A+S− reference strains (Fig. 4). Soft agar surfaces favor social gliding, whereas hard agar surfaces favor adventitious gliding (41).

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On hard agar, wild-type cells form large colonies with rafts as well as single cells at the edge (Fig. 4A). A−S+ colonies have a few rafts at the edge. In contrast, A−S− cells are nonmotile and form colonies with a sharp edge. The digR mutant cells form colonies with only a few rafts and single cells at the edge. Introduction of the digR mutation into A−S− cells results in the formation of sharp-edged colonies. Introduction of the digR mutation into A−S− cells results in the formation of colonies with only a few single cells present at the edge. On soft agar (Fig. 4B), wild-type and A−S+ cells form large colonies, A−S− cells form small colonies, and A−S− cells form nonsprawling colonies. The digR mutation gave rise to the formation of nonsprawling colonies both in the wild-type background (SA1801) and in the A−S+ (SA1803) and A−S− (SA1802) backgrounds. Consistently, examination of the behavior of digR mutant cells using time-lapse microscopy showed that single cells as well as groups of cells behaved similarly and displayed little movement. From these analyses we conclude that DigR is absolutely required for S motility and partially required for A motility.
fibrils, we analyzed the accumulation of the FibA metalloprotease, which is recognized by MAb 2105 (28) and is a dominant protein in the extracellular matrix fibrils (7), in total-cell lysates (Fig. 5D). As a negative control, we used strain DK3470, which carries a mutation in digR and is defective in synthesis of extracellular fibrils (3). To determine whether the altered level of Tfp and the altered extracellular matrix fibrils in the digR mutant have an effect on cell-cell interactions, we analyzed the ability of digR cells to agglutinate in suspension. As shown in Fig. 6, wild-type cells agglutinated whereas pilA mutant cells (DK10407) and dsp mutant cells (DK3470) were unable to agglutinate. digR cells showed an agglutination defect, which was less dramatic than that of the pilA and dsp mutants. These results support the notion that digR mutant cells have altered cell surface properties.

Global analysis of genes differentially expressed in digR cells. Little is known about the molecular components required for A motility. DigR likely acts as a transcriptional regulator. Therefore, to further the understanding of how DigR may cause defects in the A and S motility systems, we performed genome-wide expression profiling experiments to identify genes that are directly or indirectly regulated by DigR. For these experiments, we used an M. xanthus DNA microarray covering 80% of the 7,388 ORFs on the M. xanthus genome (available at www.tigr.org) (see Materials and Methods). Each ORF is represented on the microarray as a 275- to 325-bp PCR fragment. Total RNA was isolated from mid-exponentially growing wild-type (DK1622) and ΔdigR (SA1804) cells, and cDNA was prepared, labeled with Cy3 or Cy5, and competitively hybridized to the microarray. A total of five biological experiments were performed, and for each biological experiment two hybridizations were carried out as dye reversals. Thus, data analysis (see Materials and Methods) was carried at significantly higher levels than wild-type cells, whereas the negative control, DK3470, which carries the dsp mutation and is deficient in synthesis of the extracellular fibrils, bound the two dyes at reduced levels. From these analyses, we conclude that DigR is important for correct accumulation of the polysaccharide portion of the extracellular matrix fibrils.

Table 2. Binding of Congo red and trypan blue

<table>
<thead>
<tr>
<th>Strain (genotype)</th>
<th>% of dye bound (mean ± SD)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Congo red</td>
</tr>
<tr>
<td>DK1622 (wild type)</td>
<td>41.4 ± 5.9</td>
</tr>
<tr>
<td>SA1801 (digR::pMO70)</td>
<td>57.6 ± 3.9</td>
</tr>
<tr>
<td>DK3470 (dsp1693)</td>
<td>11.4 ± 2.2</td>
</tr>
</tbody>
</table>

*The percentage of dye bound by each sample was calculated from the ratio obtained by dividing the absorbance of each sample by the absorbance of the control without any cells added. Experiments were carried out in triplicate.
out on 10 experimental values for each gene. The ratio-normalized data set was analyzed using Acuity, version 4.0 (Axon Instruments), and SAM v.2.0, which assigns a score to each feature on a microarray on the basis of changes in gene expression relative to the standard deviation of repeated measurements (52). Genes called significantly regulated (directly or indirectly) by DigR were selected by a delta value of the SAM analysis where the false discovery rate became 0% in combination with a threefold cutoff criterion. From these analyses, 118 genes were identified that were significantly up- or down-regulated in the digR mutant. These genes exhibited mean expression ratios in the range of 3- to 92-fold. The digR-dependent changes in gene expression were asymmetric: 88 genes were expressed at significantly lower levels in the digR mutant, and 30 genes were expressed at significantly higher levels in the digR mutant. (The entire list of differentially expressed genes is given in Table S1 in the supplemental material.)

To validate the significance of the expression data obtained from the DNA microarrays, quantitative real-time PCR analysis was applied to 20 genes (9 genes with significantly higher expression levels in the wild type; 5 genes with significantly higher expression levels in the digR mutant; and 6 genes expressed at the same levels in both strains). The transcriptional differences determined in the microarray experiments were confirmed by the quantitative real-time PCR analyses (Table 3; see also Table S1 in the supplemental material).

Functional classification of genes differentially expressed in digR cells. Sixty-two of the 118 differentially expressed genes encode proteins for which a function can be predicted based on similarity to other proteins or which contain a conserved domain; 16 encode conserved hypothetical proteins; and 40 encode hypothetical proteins. (Table 3 lists the 62 differentially expressed genes that encode proteins with a predicted function or a conserved domain.) Among the 118 differentially expressed genes, we identified only 2 genes that have previously been implicated in A or S motility: agmQ, which encodes an aminopeptidase homolog and is required for A motility (64), is 3.7-fold down-regulated in the digR mutant, and cheY4, which encodes a single domain response regulator and is required for S motility, is 3.6-fold down-regulated in the digR mutant (53).

Consistent with the observation that DigR is required for normal synthesis of the extracellular matrix fibrils, we found that among the 118 differentially expressed genes, 42 (36%) are predicted to encode exported proteins based on the presence of a signal peptide, a twin-arginine signal sequence, one or more predicted membrane-spanning segments, and/or homology to outer membrane proteins. Among the 62 proteins with a predicted function or a conserved domain, 24 (39%) are likely to be exported.

Thirteen putative proteases, including the FibA metalloprotease, make up the largest category of proteins for which a putative function could be assigned. Nine of the proteases are likely to be secreted. Fourteen genes likely encode hydrolases, one of which is predicted to be secreted. Six genes likely encode transcription factors. This group includes four ECF sigma factors. In addition, four genes are likely to encode signal transduction proteins.

**DISCUSSION**

Here we have analyzed the function of the DigR protein, which consists of an N-terminal receiver domain and a C-terminal DNA binding domain of the HTH_XRE/HTH_3 type. Functional analyses of a digR mutant showed that DigR is absolutely required for Tfp-dependent motility, partially required for A motility, and absolutely required for fruiting body formation. Mutants with deficiencies in the A or type IV pilus-dependent-motility system often show defects in fruiting body formation (22). Therefore, we suggest that the primary functional defects in the digR mutant are the motility defects and that the defect in fruiting body formation is a secondary effect of the motility defects. Genetic evidence suggests that DigR function depends on phosphorylation of the phosphorylatable aspartate residue, D53, in the receiver domain. Thus, genetically, DigR qualifies as a response regulator. Analyses of other response regulators in the SMART database (40) showed that DigR is the first identified response regulator with an HTH_XRE/HTH_3 output domain. Thus, DigR defines a novel subfamily of response regulators of two-component signal transduction proteins.

To characterize the mechanism underlying the motility defects in a digR mutant, we used two experimental approaches. In one approach, we directly analyzed the digR mutant for the presence of the three surface structures required for Tfp-dependent motility: Tfp, LPS O antigen, and the extracellular matrix fibrils. The digR mutant has slightly reduced Tfp biosynthesis, whereas LPS O-antigen synthesis is normal. Interestingly, the composition of the extracellular matrix fibrils was found to be abnormal in a digR mutant, with a strongly reduced level of the FibA metalloprotease and an increased level of the polysaccharide portion. Consistent with the reduced synthesis of Tfp and the abnormal composition of the extracellular matrix fibrils, digR mutant cells display decreased agglutination. Thus, these analyses show that DigR has a function in regulating the composition of the extracellular matrix fibrils.

Because the understanding of the A motility machinery is less advanced than that of the Tfp-dependent motility system, we used global gene expression profiling experiments using DNA microarrays as a second approach to characterize the mechanism(s) underlying the motility defects in a digR mutant. The rationale for this experimental approach is that DigR is likely to act as a transcriptional regulator. Thus, global gene profiling experiments were likely to lead to identification of genes directly or indirectly regulated by DigR. These analyses identified 118 genes that are differentially expressed in exponentially growing digR cells compared to exponentially growing wild-type cells. Interestingly, among the genes previously identified to be important for A and Tfp-dependent motility, only two were found to be differentially expressed in a digR mutant. The A motility gene agmQ, which encodes an aminopeptidase homolog (64), is expressed at a 3.7-fold-lower level in the digR mutant; and the cheY4 gene, which encodes a single domain response regulator and which has been suggested to modulate the activity of Tfp (53), is expressed at a 3.6-fold-lower level in the digR mutant. Notably, the following genes, which are required for Tfp-dependent motility and have been implicated in synthesis of either Tfp or extracellular matrix fibrils, were expressed at similar levels in digR mutant and wild-type cells: the...
### Proteases
- MXAN0100: Putative aminopeptidase
- MXAN4410: Putative cephalosporin hydroxylase family protein
- MXAN3488: Putative trypsin-like peptidase
- MXAN1672: HTX exoprotein-like protein
- MXAN4290: Putative thioesterase
- MXAN1876: Protein required for fruiting body formation
- MXAN5466: Putative tryptophylpeptidase
- MXAN3721: Putative caspase-like protease
- MXAN1967: Putative subtilisin-like protease
- MXAN2938: Putative chitin binding protein
- MXAN4292: Putative polyketide synthase
- MXAN2188: Putative glucose dehydrogenase
- MXAN3113: fruC

### Hydrolyses
- MXAN5428: Putative metallo-β-lactamase
- MXAN5488: Putative β-hydrolase
- MXAN4295: Putative patulin-like phospholipase
- MXAN3488: Putative trypsin-like peptidase
- MXAN4419: Putative β-hydrolase
- MXAN4935: Putative esterase
- MXAN4746: Putative outer membrane protein
- MXAN5274: ATPase, histidine kinase, DNA gyrase B, and HSP90-like domain protein
- MXAN2574: RNase HII
- MXAN0221: Putative lipase

### Miscellaneous
- MXAN3113: fruC
- MXAN1672: RTX exoprotein-like protein
- MXAN5040: Putative aldehyde dehydrogenase
- MXAN7124: RTX exoprotein-like protein
- MXAN2188: Putative glucose dehydrogenase
- MXAN5074: Putative stage V sporulation protein G
- MXAN6931: Putative ECF sigma factor
- MXAN2574: RNase HII
- MXAN0221: Putative lipase

### Table 3: Selected genes differentially expressed in digR mutant cells compared to wild-type cells

<table>
<thead>
<tr>
<th>TIGR ORF</th>
<th>Gene name</th>
<th>Annotation</th>
<th>Expression ratio (mean ± SEM) on microarrays</th>
<th>RT-PCR result</th>
<th>Likely location</th>
</tr>
</thead>
<tbody>
<tr>
<td>MXAN0100</td>
<td>fibrA</td>
<td>Matrix-associated zinc metalloprotease</td>
<td>−16.5 ± 2.2</td>
<td>−</td>
<td>E</td>
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<tr>
<td>MXAN4410</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>MXAN4290</td>
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<tr>
<td>MXAN1876</td>
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<tr>
<td>MXAN3113</td>
<td>fruC</td>
<td>Protein required for fruiting body formation</td>
<td>−38.4 ± 12.8</td>
<td>E</td>
<td></td>
</tr>
<tr>
<td>MXAN1672</td>
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<td>MXAN5040</td>
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<td>MXAN7124</td>
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<td>MXAN2188</td>
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<td>MXAN5074</td>
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<td>MXAN6931</td>
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Continued on following page
TABLE 3—Continued

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<thead>
<tr>
<th>TIGR ORF</th>
<th>Gene name</th>
<th>Annotation</th>
</tr>
</thead>
<tbody>
<tr>
<td>MXAN4796</td>
<td>Fibronectin type III domain protein</td>
<td>Expression ratio&lt;sup&gt;a&lt;/sup&gt; (mean ± SEM) on microarrays: −3.7 ± 0.4 C</td>
</tr>
<tr>
<td>MXAN0575</td>
<td>Putative arsenate reductase</td>
<td>−4.1 ± 0.3 C</td>
</tr>
<tr>
<td>MXAN3638</td>
<td>Putative sphingolipid ceramide N-deacylase</td>
<td>−3.5 ± 0.5 E</td>
</tr>
<tr>
<td>MXAN4475</td>
<td>Putative DEAD/DEAH box helicase</td>
<td>−3.4 ± 0.3 C</td>
</tr>
<tr>
<td>MXAN2851</td>
<td>Proprotein convertase-containing protein</td>
<td>−3.3 ± 0.4 C</td>
</tr>
<tr>
<td>MXAN0962</td>
<td>Putative extracellular calcium binding protein</td>
<td>−3.1 ± 0.4 C</td>
</tr>
<tr>
<td>MXAN1427</td>
<td>Putative glutathione S-transferase</td>
<td>−3.0 ± 0.5 C</td>
</tr>
<tr>
<td>MXAN7037</td>
<td>Putative chemotaxis MotB protein</td>
<td>−3.0 ± 0.2 E</td>
</tr>
<tr>
<td>MXAN7299</td>
<td>Putative fatty acid desaturase</td>
<td>+14.7 ± 2.8 + 1M</td>
</tr>
<tr>
<td>MXAN6392</td>
<td>Putative acyl carrier protein</td>
<td>+3.2 ± 0.4 E</td>
</tr>
</tbody>
</table>

<sup>a</sup> Expression ratios are calculated as the expression in digR<sup>−</sup> cells over the expression in digR<sup>+</sup> cells. Thus, a negative expression ratio corresponds to a gene that is more highly expressed in the wild type than in the digR<sup>−</sup> mutant, i.e., it is positively regulated by DigR. A positive expression ratio corresponds to a gene that is more highly expressed in the digR<sup>−</sup> mutant than in the wild type, i.e., DigR negatively regulates the gene.

<sup>b</sup> Quantitative real-time PCR (RT-PCR) was performed on selected genes. Plus signs indicate genes that are more highly expressed in the wild type than in the digR<sup>−</sup> mutant; minus signs indicate genes that are more highly expressed in the digR<sup>−</sup> mutant than in the wild type.

<sup>c</sup> Proteins were classified as follows: cytoplasmic proteins (C) contain no signal peptide, no twin-arginine signal sequence, and no transmembrane domains and show homology to outer membrane proteins. Proteins were classified as follows: cytoplasmic proteins (C) contain no signal peptide, no twin-arginine signal sequence, and no transmembrane domains and show homology to outer membrane proteins; inner membrane proteins (IM) contain a predicted transmembrane domain; extracytoplasmic proteins (E) have a signal peptide predicted using SignalP (33) but no predicted transmembrane domain or homology to outer membrane proteins; outer membrane proteins (OM) show homology to outer membrane proteins.

Even though FibA is a dominant protein in extracellular matrix fibrils, it is required neither for A nor for Tfp-dependent motility (7, 28). The only defect associated with loss of FibA function is an inability to respond to dilauroyl phosphatidyl ethanolamine with a decrease in the reversal frequency (28).

The data from the global gene profiling experiments strongly suggest that DigR is not a global regulator of (known) A and Tfp-dependent motility genes. Nonetheless, these experiments revealed 118 genes in total that are differentially expressed in exponentially growing digR cells compared to exponentially growing wild-type cells. Interestingly, 36% of the 118 genes are likely to encode exported proteins, and 4 genes encode ECF sigma factor homologs. Typically, ECF sigma factors control expression of genes involved with aspects of cell envelope physiology (19). The large fraction of genes expressed in a digR-dependent manner that are likely to encode exported proteins, together with the observation that four genes encoding ECF sigma factors are differentially expressed in the digR mutant, leads us to speculate that an important function of DigR is to contribute to cell envelope homeostasis. According to this model, in a digR mutant, the expression of genes directly regulated by DigR is abnormal, and therefore, cell envelope homeostasis is perturbed. Consequently, expression of the four ECF sigma factor genes changes in an attempt to compensate for the initial perturbations. To test this model, we are currently identifying genes that are directly regulated by DigR.

Interestingly, in *E. coli*, three systems function to maintain cell envelope homeostasis: the σ<sup>E</sup> pathway (1) and the CpxAR (37) and BaeSR (36) two-component regulatory systems. σ<sup>E</sup> and the Cpx system control the expression of periplasmic protein folding catalysts and proteases, most notably DegP, whereas the function of the BaeSR targets remains to be characterized. Among the 118 genes directly or indirectly regulated...
by DigR, none are likely to encode proteins involved in protein folding in the periplasm and none encode a DegP homolog. Thus, it is not likely that DigR is a functional homolog of the DNA binding response regulator CpxR.

Approximately 40% of the response regulator genes in the *M. xanthus* genome are encoded by orphan genes, i.e., genes that are not flanked by genes encoding histidine protein kinases. digR is not flanked by a gene encoding a histidine protein kinase. Moreover, genetic evidence suggests that DigR depends on phosphorylation of the conserved, phosphorylable aspartate residue in the receiver domain for activity. Thus, genetically, DigR qualifies as a bona fide orphan response regulator. The cognate histidine protein kinase of DigR remains to be identified. If the function of DigR, as suggested, is to contribute to cell envelope homeostasis, the cognate kinase is likely membrane bound and monitors some aspect of cell envelope physiology.

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