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

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Received 5 February 2006/Accepted 24 March 2006

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

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† Supplemental material for this article may be found at <http://jb.asm.org/>.

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 146 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 a new 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' *lacZΔM15 deoR recA1 araD139 Δ(ara-leu)7697 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 CF agar (44) as described elsewhere (47). For motility assays, cells were grown in CTT broth to a density of 7×10^8 /ml, harvested, and resuspended in 1% CTT to a calculated density of 7.0×10^9 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 MZ8) 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 Mx6f and Mx6r, and the PCR product was digested with HindIII and cloned into pBGS18 (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-13f and odigR-13r. The PCR product was digested with EcoRI and XbaI and cloned into pBJ113 (24) to generate pMO95. In parallel, a 1-kb fragment of DNA downstream of *digR* was amplified using primers odigR-16f and odigR-4r and cloned into pMO95 using XbaI and HindIII to create pMO97, which carries a deletion of *digR* from position -21 to +580. pMO97 was introduced into DK1622 by electroporation, and a mutant containing a *digR* deletion was isolated as described elsewhere (24). SA1806, SA1807, SA1805, and SA1808 were constructed by integration of plasmids pMO86, pMO99, pSWU30, and pMO102, respectively, in the Mx8 phage *attB* attachment site in SA1801. pMO86, pMO99, and pMO102 (see Fig. 1) are derivatives of pSWU30 (S. Wu, personal communication) that contain the Mx8 *attP* attachment site. SA1802 and SA1803 were constructed by Mx4-mediated generalized transduction into DK1218 and

TABLE 1. Bacterial strains used in this study

Strain	Genotype	Reference
DK1622	Wild type	25
DK1218	<i>cglB2</i>	22
DK1259	<i>aglB1 sglG1</i>	22
DK1300	<i>sglG1</i>	22
DK3470	<i>dsp1693</i>	42
DK10407	<i>pilA::Tc^r</i>	55
HK1321	<i>wzm</i>	16
SA1801	<i>digR::pMO70</i>	This study
SA1802	<i>cglB2 digR::pMO70</i>	This study
SA1803	<i>sglG1 digR::pMO70</i>	This study
SA1804	<i>ΔdigR</i>	This study
SA1805	<i>digR::pMO70 attB::pSWU30</i>	This study
SA1806	<i>digR::pMO70 attB::pMO86</i>	This study
SA1807	<i>digR::pMO70 attB::pMO99</i>	This study
SA1808	<i>digR::pMO70 attB::pMO102</i>	This study

DK1300 using Mx4 phages propagated on SA1801 (13). All fragments generated by PCR were checked by DNA sequencing. All strains were checked by Southern blotting or by PCR.

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 pBGS18 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 pMO84 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 pBJ113 in two steps. First, a PCR fragment was generated using primers odigR-15f and odigR-15r. The resulting PCR fragment was digested with HindIII and XbaI and cloned into pBJ113 to yield pMO96. Next, a PCR fragment of *digR* was amplified using primers odigR-16f and odigR-1r. The resulting PCR fragment was digested with XbaI and EcoRI and cloned into pMO96 to yield pMO98. This construct was moved into pSWU30 to yield pMO99. The aspartate residue D53 in DigR was replaced by an asparagine by changing the aspartate 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 corresponding mutation. This fragment was digested with XbaI and EcoRI and 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.

Congo red and trypan blue binding assays. To quantify binding of Congo red or trypan blue, a liquid binding assay was adapted from the work of Black and Yang (10) except that 5×10^8 cells from mid-exponential cultures were harvested, washed, and resuspended in 900 μl of 10 mM morpholinepropanesulfonic acid (MOPS) (pH 7.6)–2 mM MgSO₄ buffer.

Immunoblot analysis. To determine the amount of PilA assembled in Tfp, Tfp were sheared off from cells as described elsewhere (60). Immunoblotting was carried out as described elsewhere (39) using rabbit anti-PilA antibodies (60). For analyses of total PilA and FibA levels, the immunoblots were probed either with anti-PilA antibodies or with the anti-FibA monoclonal antibody (MAb) 2105 (28). The secondary antibodies used were either horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (for PilA) or horseradish peroxidase-conjugated rabbit 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 stop solution (1/10 culture 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.

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 MicroarrayArchitect (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 postspotted before use as described elsewhere (12) with the following modifications. Slides were rehydrated in boiling filtered, deionized 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 amines, 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 CyScribe postlabeling kit (Amersham Biosciences) according to the protocol provided by the manufacturer. Probes were mixed with final concentrations of 3× SSC, 0.5 µg/µl yeast tRNA (Sigma), and 0.3% SDS. The hybridization solution was boiled at 100°C for 2 min and cooled at room temperature for 5 min before application to a slide. Hybridizations were carried out under a 22- by 40-mm lifter slip in a total volume of 60 µl. The hybridization chamber was assembled, sealed, and submerged in a water bath at 60°C for 5 to 16 h. After hybridization, slides were washed for 5 min in 2× SSC–0.1% SDS, for 5 min in 0.2× SSC–0.1% SDS, and for 5 min in 0.2× SSC, followed by a short rinse in H₂O. Slides were dried by centrifugation and scanned immediately.

Data acquisition and analysis. Microarrays were scanned separately 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 v2.0), 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 ("Flags" ≥ 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 v2.0 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 80% 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

FIG. 1. Physical map of the *digR* region. (A) Schematic representation of the *digR* locus. ORFs located upstream and downstream from *digR* are indicated by the arrows. Arrows indicate the direction of transcription. Coordinates are relative to the start codon of *digR*. The deduced proteins of the ORFs flanking *digR* have the following characteristics: MXAN5589 lacks similarity to database entries; MXAN5591 is similar to rhomboid family proteins; MXAN5593 is a forkhead-associated domain- and tetratricopeptide repeat-containing protein; MXAN5594 is similar to enoyl coenzyme A hydratases/isomerases; MXAN5595 is similar to biotin carboxyl carrier proteins; MXAN5596 is similar to acetyl coenzyme A carboxylases. The MXAN numbering of ORFs is adapted from The Institute for Genomic Research's Comprehensive Microbial Resource (<http://cmr.tigr.org>). (B) Schematic representation of plasmids used in this work. Plasmids pMO70, pMO97, pMO86, and pMO99 contain *M. xanthus* DNA fragments as indicated. pMO102 contains the *digR*(D53N) allele.

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_XRE and HTH_3, 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 pBGS18. 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

FIG. 2. DigR represents a new subfamily of response regulator proteins. (A) Alignment of N-terminal receiver domain with the receiver domains of Rrp2 from *Borrelia burgdorferi* (30% identity, 60% similarity), SaeR from *Staphylococcus aureus* (30% identity, 60% similarity), and ZraR from *Klebsiella oxytoca* (37% identity, 60% similarity). Asterisks indicate conserved signature residues of receiver domains (49). Residues on black, dark gray, and light gray backgrounds are 100, 75, and 50% conserved, respectively. (B) Alignment of the C-terminal DNA binding domain of DigR with the DNA binding domains in the Cro repressor protein of bacteriophage 434 (18% identity, 34% similarity), the CI repressor protein of bacteriophage 434 (14% identity, 28% similarity), and the SinR protein of *Bacillus subtilis* (18% identity, 33% similarity). Asterisks indicate the glycine residues in the turn and hydrophobic residues that are important for maintaining the arrangement of the two helices in the HTH motif (34). The helix-turn-helix motif is indicated schematically. Conserved residues are indicated as for panel A. The alignments were made using CLUSTAL (51).

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, *aitB*, on the chromosome. Plasmid pMO86 contains a 4,826-bp fragment that includes *digR* and the two ORFs upstream of *digR* (Fig. 1). Introduction

FIG. 3. Colony morphology and developmental phenotypes of *digR* mutants. For colony morphology (upper row), the indicated strains were incubated at 32°C for 24 h on 0.5% CTT–1.5% agar medium and viewed with a Leica MZ8 stereomicroscope. Bar, 1 mm. For the developmental phenotypes (lower row), cells were incubated for 120 h on CF agar at 32°C. Bar, 0.5 mm. Veg., vegetative; Starv., starving.

FIG. 4. Motility defects caused by *digR* mutation. (A) Motility phenotype of *digR* cells on 1.5% agar. Strains used are DK1622, DK1218, DK1300, and DK1259 (left column) and SA1801, SA1802, and SA1803 (right column). Scale bar: 5 mm. (B) Motility phenotype of *digR* cells on 0.5% agar. Strains used are as in panel A. Cells were incubated at 32°C for 24 h and then viewed with a Leica MZ8 stereomicroscope.

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 $\Delta digR$ 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 adventurous 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 form colonies with many single cells at the edge. 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 nonspreading colonies. The *digR* mutation gave rise to the formation of nonspreading 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^r strain, DK10407, as a negative control. As shown in Fig. 5A, the total level of PilA in the *digR* mutant is slightly higher than that in wild-type cells. To determine the amount of PilA assembled into Tfp, Tfp were sheared off from cells and purified as described elsewhere (60), and proteins were separated by SDS-polyacrylamide gel electrophoresis, followed by immunoblot analyses with the anti-PilA antibodies. As shown in Fig. 5B, approximately twofold less PilA is present in Tfp in the *digR* mutant than in wild-type cells. We conclude that *digR* cells assemble Tfp, although at a lower level than wild-type cells.

We determined the accumulation of O antigen in immunoblot blots of whole cells using a monoclonal antibody (MAB 783) directed against the O antigen (15), with strain HK1321, which carries a mutation in the *wzm* gene, as a negative control. *wzm* encodes a subunit of an ABC transporter required for synthesis of the O antigen. As shown in Fig. 5C, *digR* mutant cells accumulated O antigen at a level similar to that of wild-type cells.

To examine the protein portion of the extracellular matrix

FIG. 5. Accumulation of cell surface structures required for S motility. (A) Immunoblot of total PilA accumulation in DK1622 (wild type), SA1801 (*digR*), and DK10407 (*pilA*) cells. The same amount of total protein from exponentially grown cultures was loaded in each lane. (B) Immunoblot of PilA assembled into type IV pili. Pili were sheared off from exponentially growing cells. The same amount of total protein was loaded in each lane. Strains are as in panel A. (C) Immunoblot of whole cells reacted with MAb 783, specific to the O-antigen portion of LPS. The same number of cells was loaded in each slot. Strains are as in panel A except that the *wzm* strain, which is deficient in O-antigen biosynthesis, is HK1321. (D) Immunoblot of total accumulation of the FibA metalloprotease. The same amount of total protein from exponentially grown cultures was loaded in each lane. Strains are as in panel A except that the *dsp* strain, which is deficient in *fibA* synthesis, is DK3470. The positions of molecular size standards and their sizes (in kilodaltons) are marked on the left.

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 *dsp* and is defective in synthesis of extracellular matrix fibrils. As reported previously, MAb 2105 recognizes two bands with apparent sizes of 66 kDa and 31 kDa in wild-type cells, corresponding to the mature form of FibA and a processed form of FibA (28). In the *digR* mutant (SA1801), the 66-kDa protein was barely detected and the 30-kDa protein was not detected. Thus, DigR is required for accumulation of FibA.

The polysaccharide portion of the extracellular matrix fibrils was examined by analyzing the binding of Congo red and trypan blue to whole cells. These two dyes bind to the polysaccharide portion of extracellular matrix fibrils (4, 14). As shown in Table 2, *digR* cells bound Congo red and trypan blue

TABLE 2. Binding of Congo red and trypan blue

Strain (genotype)	% of dye bound (mean $\pm$ SD) ^a	
	Congo red	Trypan blue
DK1622 (wild type)	41.4 $\pm$ 5.9	18.5 $\pm$ 3.8
SA1801 (<i>digR</i> ::pMO70)	57.6 $\pm$ 3.9	37.9 $\pm$ 5.7
DK3470 (<i>dsp1693</i>)	11.4 $\pm$ 2.2	3.9 $\pm$ 1.5

^a 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.

FIG. 6. Cell agglutination assay. Agglutination was measured by monitoring the decrease in absorbance at 625 nm of a suspension of mid-exponentially growing cells in agglutination buffer. The relative absorbance was calculated by dividing the absorbance measured at each time point by the initial absorbance for each strain. The assay was carried out in triplicate for each strain. The data plotted are average values for three replicates.

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.

***digR* cells exhibit altered cell-cell interactions.** Agglutination of wild-type *M. xanthus* cells depends on Tfp (58) and the extracellular matrix 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 $\Delta digR$ (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

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 hydroxylases, 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

TABLE 3. Selected genes differentially expressed in *digR* mutant cells compared to wild-type cells

TIGR ORF	Gene name	Annotation	Expression ratio ^a (mean $\pm$ SEM) on microarrays	RT-PCR result ^b	Likely location ^c
Proteases					
MXAN0100	<i>fibA</i>	Putative aminopeptidase	-16.5 $\pm$ 2.2	—	E
MXAN1650		Putative trypsin-like peptidase	-16.5 $\pm$ 1.5		C
MXAN6106		Matrix-associated zinc metalloprotease	-12.3 $\pm$ 2.1	—	E
MXAN5970		Putative subtilisin-like protease	-12.1 $\pm$ 2.3		E
MXAN5392		Putative zinc-dependent metalloprotease	-9.4 $\pm$ 2.2		C
MXAN2127	<i>agmQ</i>	Putative zinc metalloproteinase	-6.6 $\pm$ 1.6	—	E
MXAN3676		Putative metalloprotease	-6.5 $\pm$ 0.7	—	E
MXAN1967		Putative subtilisin-like protease	-3.9 $\pm$ 0.6		E
MXAN2923		Putative aminopeptidase	-3.7 $\pm$ 0.4	—	C
MXAN5466		Putative trypsin-like peptidase	-3.1 $\pm$ 0.7		C
MXAN5454		Putative metallopeptidase	+6.7 $\pm$ 1.9		E
MXAN6352		Putative serine protease	+3.2 $\pm$ 0.2		E
MXAN7216		Putative caspase-like protease	+3.1 $\pm$ 0.7		E
Transcription factors					
MXAN7289		Putative ECF sigma factor	-40.6 $\pm$ 9.5	—	C
MXAN2230		Putative transcriptional regulator	-13.7 $\pm$ 1.8		C
MXAN7214		Putative ECF sigma factor	+14.3 $\pm$ 3.7	+	C
MXAN6058		Putative ECF sigma factor	+6.6 $\pm$ 1.4		C
MXAN1093		DNA binding response regulator	+3.6 $\pm$ 0.3	+	C
MXAN0233		Putative ECF sigma factor	+3.4 $\pm$ 0.7		C
Signal transduction proteins					
MXAN4202	<i>cheY4</i>	Single domain response regulator	-6.8 $\pm$ 3.3		C
MXAN4841		Putative Ser/Thr protein kinase	-5.5 $\pm$ 0.9		C
MXAN2684		Response regulator	-3.6 $\pm$ 0.6	—	C
MXAN3668		Methyl-accepting chemotaxis protein	-3.4 $\pm$ 0.4	—	IM
Outer membrane proteins					
MXAN4746		Putative outer membrane protein	-16.5 $\pm$ 1.6		OM
MXAN6911		Putative TonB-dependent siderophore receptor	+4.8 $\pm$ 0.6	+	OM
Membrane transport proteins					
MXAN5379		Putative sugar ABC transporter	-3.8 $\pm$ 0.7		IM
MXAN4294		Putative anion-transporting ATPase	-3.6 $\pm$ 1.0		C
MXAN1671		Putative V-type H ⁺ -translocating pyrophosphatase	+4.5 $\pm$ 1.0		IM
Hydroxylases					
MXAN5428		Putative metallo- β -lactamase	-26.7 $\pm$ 8.3		C
MXAN3488		Putative β -hydroxylase	-7.1 $\pm$ 1.0		C
MXAN4295		Putative patatin-like phospholipase	-5.8 $\pm$ 1.7		C
MXAN4290		Putative thioesterase	-4.6 $\pm$ 1.1		C
MXAN4419		Putative β -hydroxylase	-4.5 $\pm$ 1.0		C
MXAN4935		Putative esterase	-4.3 $\pm$ 0.8		C
MXAN1744		Putative lipoygenase	-4.1 $\pm$ 0.7		C
MXAN4303		Putative type I phosphodiesterase	-3.3 $\pm$ 0.7		C
MXAN4410		Putative cephalosporin hydroxylase family protein	-3.1 $\pm$ 0.2		C
MXAN0366		Endonuclease/exonuclease/phosphatase family protein	+6.5 $\pm$ 1.0		C
MXAN6394		Putative metallo- β -lactamase	+4.8 $\pm$ 0.6		C
MXAN2749		ATPase, histidine kinase-, DNA gyrase B-, and HSP90-like domain protein	+4.0 $\pm$ 0.7		C
MXAN2574		RNase HII	+3.8 $\pm$ 1.3		C
MXAN0221		Putative lipase	+3.5 $\pm$ 0.9		E
Miscellaneous					
MXAN3113	<i>fruC</i>	Protein required for fruiting body formation	-38.4 $\pm$ 12.8		E
MXAN1672		RTX exoprotein-like protein	-11.6 $\pm$ 1.7		E
MXAN5040		Putative aldehyde dehydrogenase	-11.2 $\pm$ 4.1		C
MXAN7124		Putative iron-sulfur cluster-binding protein, Rieske family	-9.5 $\pm$ 4.2		C
MXAN2188		Putative glucose dehydrogenase	-5.9 $\pm$ 0.6		C
MXAN5074		Putative stage V sporulation protein G	-5.6 $\pm$ 0.3		C
MXAN6679		Putative Rhs family protein	-5.4 $\pm$ 0.8		E
MXAN2938		Putative chitin binding protein	-4.7 $\pm$ 0.6		E
MXAN4130		WD domain protein	-4.6 $\pm$ 1.1		C
MXAN4292		Putative polyketide synthase	-3.5 $\pm$ 0.9		C

Continued on following page

TABLE 3—Continued

TIGR ORF	Gene name	Annotation	Expression ratio ^a (mean $\pm$ SEM) on microarrays	RT-PCR result ^b	Likely location ^c
MXAN4796	Fibronectin type III domain protein		-3.7 ± 0.4		C
MXAN0575	Putative arsenate reductase		-4.1 ± 0.3		C
MXAN3638	Putative sphingolipid ceramide <i>N</i> -deacylase		-3.5 ± 0.5		E
MXAN4475	Putative DEAD/DEAH box helicase		-3.4 ± 0.3		C
MXAN2851	Proprotein convertase-containing protein		-3.3 ± 0.4		C
MXAN0962	Putative extracellular calcium binding protein		-3.1 ± 0.4		E
MXAN1427	Putative glutathione <i>S</i> -transferase		-3.0 ± 0.5		C
MXAN7037	Putative chemotaxis MotB protein		-3.0 ± 0.2		E
MXAN7299	Putative fatty acid desaturase		$+14.7 \pm 2.8$	+	IM
MXAN6392	Putative acyl carrier protein		$+3.2 \pm 0.4$		E

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

^b 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 $digR$ mutant; minus signs indicate genes that are more highly expressed in the $digR$ mutant than in the wild type.

^c Proteins were classified as follows: cytoplasmic proteins (C) contain no signal peptide, no twin-arginine signal sequence, and no transmembrane domains and show no 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.

pil genes, which encode proteins involved in Tfp biogenesis and function (54, 58, 59, 61, 62); the *dif* genes, which encode a signal transduction system required for synthesis of the extracellular matrix fibrils (8, 10, 63); the *sglK* gene, which encodes an Hsp70 homolog required for synthesis of the extracellular matrix fibrils (56); the *eps* genes, which encode enzymes involved in synthesis of the polysaccharide portion of the extracellular matrix fibrils (31); and the *rasA* gene, which is required for synthesis of the extracellular matrix fibrils (35). Likewise, expression of the three genes (*nla24* and *mglAB*) that are required for both the A and S motility systems was similar in $digR$ and wild-type cells.

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 phosphatidylethanolamine with a decrease in the reversal frequency (28). Likewise, an increased content of polysaccharide in the extracellular matrix fibrils has previously been shown not to cause defects in A and Tfp-dependent motility (10). On the basis of the available data, we suggest that the partial A motility defect in a $digR$ mutant is at least in part due to decreased expression of *agmQ*. The defect in Tfp-dependent motility could be caused at least in part by the combined effect of decreased expression of *cheY4* and the slightly decreased level of Tfp. In addition, since the assembly and function of Tfp, as well as of the nozzle-like structures implicated in A motility, conceivably depends on an intact cell envelope, it remains a possibility that the altered cell envelope in a $digR$ mutant (see below) may contribute to the motility defects.

To our knowledge, the defect in the composition of the extracellular matrix fibrils (decreased FibA accumulation and increased accumulation of exopolysaccharides) observed in a $digR$ mutant has not been reported previously. This may suggest that *digR* is part of a signal transduction pathway that operates independently of the *dif*, *nla24*, *sglK*, and *rasA* pathways to regulate synthesis of the extracellular matrix fibrils. In the global gene profiling experiments, *fibA* was expressed at a

12.3-fold-lower level in the $digR$ mutant than in the wild type, suggesting that *digR* directly or indirectly regulates expression of *fibA*. Among the genes that are differentially expressed in a $digR$ mutant, none are predicted to be involved in exopolysaccharide biosynthesis. Therefore, the mechanism underlying the increased accumulation of exopolysaccharides in the $digR$ mutant remains to be explained. Likewise, it remains to be clarified whether the chemical composition of the polysaccharide portion of the extracellular matrix fibrils in a $digR$ mutant is different from that of wild-type cells.

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 differently 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^E pathway (1) and the CpxAR (37) and BaeSR (36) two-component regulatory systems. σ^E 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, phosphorylatable 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.

ACKNOWLEDGMENTS

We thank Allan K. Nielsen for excellent and invaluable technical advice and support, Jimmy Jakobsen for printing the first DNA microarrays, the members of the *Myxococcus* Microarray Consortium for providing the DNA microarrays used in these experiments, and Penelope Higgs for careful reading of the manuscript.

We thank Novozymes A/S for a Novo scholarship awarded to M.O. Sequencing of *M. xanthus* at TIGR was accomplished with support from the NSF. The Danish Natural Science Council supported this work.

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