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Triple Mutants Uncover Three New Genes Required for Social Motility in *Myxococcus xanthus*

Philip Youderian* and Patricia L. Hartzell[†,1]

**Department of Biology, Texas A&M University, College Station, Texas 83843-3052 and*
[†]*Department of Microbiology, Molecular Biology and Biochemistry, University of Idaho, Moscow, Idaho 83844-3052*

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ABSTRACT

The bacterium *Myxococcus xanthus* glides over surfaces using two different locomotive mechanisms, called S (social) and A (adventurous) motility that enable cells to move both as groups and as individuals. Neither mechanism involves flagella. The functions of these two motors are coordinated by the activity of a small Ras-like protein, encoded by the *mglA* gene. The results of previous studies of a second-site suppressor of the *mglA-8* missense mutation *masK-815* indicate that MglA interacts with a protein tyrosine kinase, MasK, to control social motility. Sequence analysis of the sites of 12 independent insertions of the transposon *magellan-4* that result in the loss of motility in an *M. xanthus mglA-8 masK-815* double mutant shows that nine of these 12 insertions are in genes known to be required for S gliding motility. This result confirms that the *masK-815* suppressor restores S but not A motility. Three of the 12 insertions define three new genes required for S motility and show that the attachment of heptose to the lipopolysaccharide inner core, an ortholog of the CheR methyltransferase, and a large protein with YD repeat motifs, are required for S motility. When these three insertions are backcrossed into an otherwise wild-type genetic background, their recombinants are found to have defects in S, but not, A motility. The spectrum of *magellan-4* insertions that lead to the loss of S motility in the *mglA-8 masK-815* double mutant background is different than that resulting from a previous mutant hunt starting with a different (A mutant) genetic background, suggesting that the number of genes required for S motility in *M. xanthus* is quite large.

EUBACTERIAL genomes are extraordinarily diverse and range in size from <1 Mbp to >10 Mbp. In general, the bacteria with genomes at the larger extreme of genome size, such as species of Anabena, Myxococcus, and Streptomyces, display more complex behaviors in response to environmental stresses, including programs of multicellular development that involve the differentiation of cells into specialized forms. For example, *Myxococcus xanthus,* with its 9-Mb genome, responds to starvation by aggregating large groups of individual cells into fruiting bodies, in which a subset of cells differentiate into heat-resistant, diploid myxospores.

This response to starvation by *M. xanthus* requires the functions of a large set of genes, many of which do not have homologs in other bacteria. Within the set of genes required for the multicellular development of *M. xanthus* are two subsets of genes involved in its two different mechanisms of gliding motility. *M. xanthus* can glide over solid surfaces without the use of flagella, both as individual cells (called A, or "adventurous" motility) and as groups of cells (called S, or "social" motility). These two mechanisms can be separated genetically. Most mutations induced by chemical mutagens or transposons that impair motility in *M. xanthus* affect its ability to glide as single cells, or as groups of cells, but not both (Hodgkin and Kaiser 1979a,b; Macneil *et al.* 1994a,b; Wu and Kaiser 1995; Youderian *et al.* 2003; Youderian and Hartzell 2005).

Wild-type strains of *M. xanthus* form large, spreading colonies on agar plates. Most single mutants of *M. xanthus* with defects in either A or S motility form colonies of intermediate size. Double mutants of *M. xanthus* with pairs of mutations, one in an A motility gene plus one in an S motility gene, form smaller colonies than either single A or S mutants. These colonies have sharp edges, and the colonies formed by double mutants with both A and S defects can be distinguished readily from those made by either a wild-type strain or single A or S mutants, both on the basis of their relative size and their morphology. In the past, we have used these phenotypic differences to screen for double mutants with additional defects in A and S motility (Macneil *et al.* 1994a,b; Youderian *et al.* 2003; Youderian and Hartzell 2005).

Our most fruitful screens for mutants defective in the two motility systems have involved making double mutants using the transposons Tn*5* and *magellan-4*. Because transposons are both genetic and physical markers, genes disrupted by transposon insertions can be subcloned

[1]*Corresponding author:* 142 Life Science South, Molecular Biology and Biochemistry, University of Idaho, Moscow, ID 83844-3052.
E-mail: hartzell@uidaho.edu

rapidly, and the sequence junctions between transposons and their target genes can be determined to identify these target genes. Previously, we have employed a simple technique to screen for double mutants defective in both A and S motility with Tn*5* and *magellan-4* insertions and identify their target A and S genes. Starting with mutants defective in either A or S motility, we mutagenized these single mutants with transposons and screened for double mutants that form smaller, nonmotile colonies, because they carry second mutations (insertions) in S or A genes, respectively. Using this strategy, we have shown that the functions of at least 34 genes are required for A motility and have identified 45 of the 113 genes known to be required for S motility (Youderian *et al.* 2003; Youderian and Hartzell 2005; Hartzell *et al.* 2007).

The results of our studies, and those from other laboratories, have identified only three genes: *mglA*, (Stephens and Kaiser 1987; Stephens *et al.* 1989; Hartzell and Kaiser 1991a,b; Hartzell 1997); *agmA*, predicted to encode an amidase involved in the biogenesis of the cell wall (Youderian *et al.* 2003); and *epsI/nla24*, predicted to encode a positive activator of transcription (Caberoy *et al.* 2003; Lancero *et al.* 2004; Lu *et al.* 2005) required for both the S and A motility mechanisms.

The first of these three genes, *mglA*, encodes a 22-kD protein in the Ras family of GTPases, which behaves like the other small "G proteins" in this family, by coupling the hydrolysis of its GTP substrate with multiple protein–protein interactions that trigger signal transduction cascades (Hartzell and Kaiser 1991b; Hartzell 1997). MglA interacts with two different proteins, AglZ, a myosin-like coiled-coil protein involved in A motility (Yang *et al.* 2004; Mignot *et al.* 2007), and MasK, a protein kinase involved in S motility (Thomasson *et al.* 2002). The interactions between MglA and each of these proteins likely regulate the simultaneous operation of the S and A gliding motors of *M. xanthus*, to coordinate the actions of both motors simultaneously so that they function in the same direction.

The interaction between MglA and the protein tyrosine kinase, MasK, was discovered by using a classical genetic approach. A missense mutation in the *mglA* gene, *mglA-8*, results in a loss of *mglA* function, and impairs both A and S motility. An allele-specific suppressor of *mglA-8*, *masK-815*, also is a missense mutation. This extragenic second-site suppressor mutation maps to the 3′ end of the essential *masK* gene. Cells of the *mglA-8 masK-815* double mutant can move as groups, but not as individuals, and appear to have regained S, but not A, motility. In addition, when a plasmid subclone of the *masK* gene was used in the yeast two-hybrid selection as bait against a library carrying plasmid subclones of *M. xanthus* chromosomal DNA, subclones carrying fusions of *mglA* with the GAL*4* activation domain were recovered, confirming the interaction between MglA and MasK. MasK, when expressed in *Escherichia coli*, has tyrosine kinase activity (Thomasson *et al.* 2002). Together, these results argue that the interaction between MglA and MasK likely mirrors those between eukaryotic GTPases and MAP kinases and controls a signal transduction cascade in *M. xanthus* that, in turn, controls S motility.

MATERIALS AND METHODS

Bacterial strains and growth conditions: The *M. xanthus* strains generated in this study are derivatives of the wild-type strain DK1622 and its *mglA-8 masK-815* double mutant derivative MxH1104 (Thomasson *et al.* 2002) and are listed in Table 1 . *M. xanthus* was grown at 32° in CTPM liquid medium (1% casitone, 10 mm Tris pH 7.6, 1 mm potassium phosphate pH 7.5, 5 mm $MgSO_4$) and CTPM agar (1.5%) plates; CTPM was supplemented with kanamycin (Kan; 40 μg/ml). Plasmids were introduced into *M. xanthus* by electroporation (Kashefi and Hartzell 1995; Youderian *et al.* 2003). *E. coli* strain DH5α(λ *pir*) was used for the recovery and propagation of plasmids and the preparation of plasmid DNA. Plasmids were introduced into this strain by electroporation, and derivatives with plasmids were grown in LB medium supplemented with kanamycin (Kan; 40 μg/ml). Plasmid pMycoMar, donor of the mini-*mariner* element *magellan-4*, has been described (Rubin *et al.* 1999). Restriction endonucleases and DNA modifying enzymes were from New England Biolabs (Ipswich, MA).

Isolation and phenotypic screening of potential social motility mutants: The electroporation of MxH1104 cells with plasmid pMycoMar was performed as described (Youderian *et al.* 2003). Electroporation was used to backcross transposon insertions from the MxH1104 background into the wild-type (DK1622) background. Chromosomal DNA was prepared from strains MxH1189, 1195, and 1198 using the Easy DNA method (Invitrogen, Carlsbad, CA). Electroporation of DK1622 cells with purified chromosomal DNA (1–3 μg) was performed as described (Youderian *et al.* 2003). Strains with the *mis-189*, *mis-195*, and *mis-198* insertions in an otherwise wild-type genetic background were designated MxH1289, 1295, and 1298, respectively. In all cases, the Kan^R determinant was found to be 100% linked with a defect in S motility (see results). Electroporation mixes were plated on CTPM Kan agar and incubated for 5 days at 32°. After incubation, plates were screened visually to identify small colonies with smooth edges. Mutants were purified twice, and the phenotypes of single colonies formed by each mutant were compared after each purification step.

Cloning and sequence analysis of *M. xanthus* genomic DNA flanking *magellan-4* insertions in S genes: To subclone *magellan-4* insertions in S genes, *M. xanthus* genomic DNA was isolated from vegetative cultures of MxH1104 S:: *magellan-4* strains, cleaved with *Bss*HI, ligated, and electroporated into *E. coli* DH5α (λ *pir*) as described (Youderian *et al.* 2003; Youderian and Hartzell 2005). Plasmid DNAs with subcloned *Bss*HII fragments were isolated from Kan^R electroporants and sequenced with primers Mar1 and Mar2 (Biosource/Invitrogen), complementary to the ends of *magellan-4* (Youderian *et al.* 2003); sequencing was performed by Commonwealth Biotechnologies (Richmond, VA). BLASTn searches (Altschul *et al.* 1990) against the DK1622 genome sequence were used to identify the TA target site for each *magellan-4* insertion, given as the coordinates of the *M. xanthus* sequence available from TIGR (http://cmr.tigr.org/tigr-scripts/CMR/GenomePage.cgi?org_search=&org=gmx) (Table 1). In all cases, these searches yielded a unique dinucleotide target site of insertion without accompanying deletion or rearrangement. The probable functions of proteins encoded by target genes inactivated by *magellan-4* insertions were deduced using the CD-search

TABLE 1

Insertions of *magellan-4* that impair social gliding motility

Coordinates	Locus	Insertion	Strain[a]	Strain[b]	Gene	Isoalleles[c]
5901872–5901873	MXAN_4707	*mis-185*	MxH1185		*sgmU; rfaF*	*mis-55*
5903725–5903726	MXAN_4710	*mis-198*	MxH1198	MxH1298	*sgnG; rfaE*	
7149933–7149934	MXAN_5774	*mis-178*	MxH1178		*pilO*	*mis-78, mis-79*
7151612–7151613	MXAN_5776	*mis-168*	MxH1168		*pilM*	
7151612–7151613	MXAN_5776	*mis-182*	MxH1182		*pilM*	
8154610–8154611	MXAN_6627	*mis-181*	MxH1181		*sgnC*	*mis-52*
8200360–8200361	MXAN_6671	*mis-196*	MxH1196		*sglK*	*mis-45*
8208665–8208666	MXAN_6679	*mis-195*	MxH1195	MxH1295	*sgnH*	
8671663–8671664	MXAN_7103	*mis-189*	MxH1189	MxH1289	*sgnI*	
9064019–9064020	MXAN_7441	*mis-190*	MxH1190		*epsH*	
9072529–9072530	MXAN_7448	*mis-179*	MxH1179		*epsD*	
9072529–9072530	MXAN_7448	*mis-180*	MxH1180		*epsD*	

The 12 insertions of *magellan-4* in the *M. xanthus* genome described in this report are listed in order of their sites within the genome sequence, which can be found at: http://www.tigr.org/tigr-scripts/CMR2/GenomePage3.spl?database=gmx. Given are the gene numbers in which the insertions are situated, the allele numbers of the insertions, the strain numbers of the derivatives of strain MxH1104 with each insertion, and the gene names. The new genes identified by *magellan-4* insertions in this study, *MXAN_4710*, *MXAN_6679*, and *MXAN_7103*, have been designated *sgnG*, *sgnH*, and *sgnI*, respectively.

[a] Strain name of the original isolate containing the *mariner* insertion in *M. xanthus* MxH1104 (*mglA8 masK-815*).

[b] Strain name of the corresponding mariner insertion in the wild-type *M. xanthus* (DK1622) background.

[c] The allele numbers of insertions that were also isolated in an Δ*aglU* are listed as isoalleles (Youderian and Hartzell 2005).

program (Marchler-Bauer and Bryant 2004) available at http://www.ncbi.nlm.nih.gov/BLAST/.

Analysis of spreading motility and single cell gliding: Motility phenotypes of mutants were compared with that of the wild-type strain using spreading assays on 0.3 and 1.5% CTPM agar as described (Shi and Zusman 1993) and by microscopic examinations of colony edges. Individual cells were tracked by time-lapse videomicroscopy and analyzed using Metamorph tracking software. Cells were grown in CTPM medium, diluted to 5×10^5 cells/ml and spotted on agar pads as described elsewhere (Mignot *et al.* 2005). The agar pad containing 1% agar in CTPM was poured on a cover slip containing a 0.5-mm silicon gasket and allowed to dry for 30 min. Cells were placed on the agar pad, inverted on a glass slide, and incubated at 32° for 30 min. The cells were viewed with a Nikon FXA microscope at 20×. Images were captured using a CCD camera at 30 sec intervals for 30 min. Stacks (consecutive series of images) were created with Metamorph, and individual cells (minimum 30 cells selected at random) were tracked to quantify the rate of cell movement and cell reversal frequency.

Developmental assays: Fruiting body formation and spore production were monitored on TPM starvation medium as described elsewhere (Yang *et al.* 2004). In each of these experiments, mutants were assayed in triplicate alongside control strains DK1622 (wild-type), DK4135 (*mglA8*), and MxH1104 (*mglA8 masK815*).

RESULTS

Insertions of *magellan-4* that impair the mobility of an *mglA*-8 *masK*-815 double mutant map within old and define new genes required for S motility: To confirm the result that the *masK-815* restores S but not A motility, we mutagenized the double *mglA-8 masK-815* mutant strain, MxH1104, with transposon *magellan-4*, which confers kanamycin resistance (Kan^R). We electroporated MxH1104 with the donor, suicide plasmid pMycoMar (Rubin *et al.* 1999) and screened for triple mutants among 2000 independent Kan^R mutants of MxH1104 that form nonmotile colonies. Among these 2000 mutants, we recovered 22 that form nonmotile colonies. Chromosomal DNA was purified from these mutants, cleaved with restriction endonucleases that do not have recognition sites within the *magellan-4* element, ligated, and used to electroporate an *E. coli* host expressing the Pir protein. Because the defective *magellan-4* transposon carries the plasmid R6Kγ origin, its replication is conditionally dependent on the Pir protein. Thus, Kan^R recombinants of this *E. coli* host arising after electroporation carry plasmids with the entire *magellan-4* element and flanking *M. xanthus* chromosomal DNA (Youderian *et al.* 2003; Youderian and Hartzell 2005). Kan^R plasmids with *magellan-4* insertions were recovered from 12 of the nonmotile *M. xanthus mglA*-8 *masK*-815 *magellan-4* triple mutants, and the sequences of both of the junctions between *magellan-4* and the *M. xanthus* chromosome were determined for each insertion by using primers complementary to, and pointing outward from, the ends of *magellan-4*. These sequences were aligned with the sequence of the *M. xanthus* genome to determine the identities of the genes inactivated by *magellan-4* insertions.

Table 1 summarizes the results of this analysis. Insertions of *magellan-4* in all 12 nonmotile triple mutants are flanked by repeats of the dinucleotide TA, the preferred target for *magellan-4* insertion. Nine of the 12 insertions are found to lie within the *pilM*, *pilO*, *sglK*, *epsD*, *epsH*, *sgmU* (*MXAN_4707*), and *sgnC* (*MXAN_6627*) genes, previously shown to be required for S motility. Three

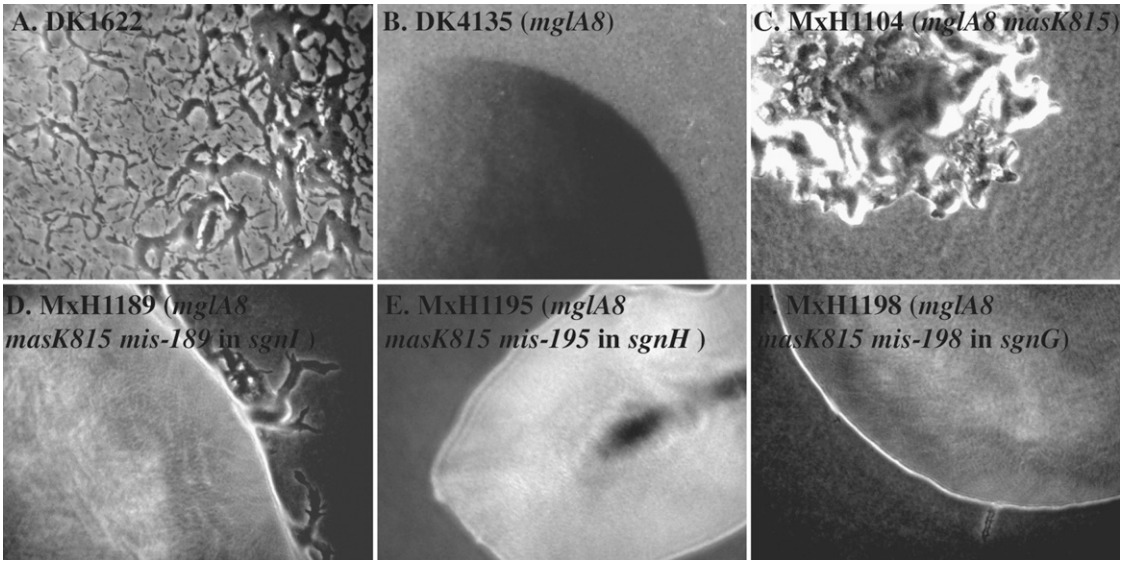

Figure 1.—Colony morphologies of mutants with *magellan-4* insertions that abolish S motility in the *mglA-8 masK-815* genetic background. Colonies formed by strains (A) DK1622 (wild type), (B) DK4135 (*mglA-8*), (C), MxH1104 (*mglA-8 masK-815*), (D) MxH1189 (*mglA-8 masK-815 mis-189*), (E) MxH1195 (*mglA-8 masK-815 mis-195*), and (F) MxH1195 (*mglA-8 masK-815 mis-198*) are shown. The *mis*-189, *mis-195,* and *mis-189* alleles are *magellan-4* insertions in the MXAN_7103, MXAN_6679, and MXAN_4710 genes, respectively (Table 1), predicted to encode a homolog of the CheR methyltransferase named SgnI, a large protein with YD repeat motifs named SgnH and an enzyme that is involved in the LPS biosynthesis pathway named SgnG. Photographs were taken using a Nikon FXA microscope, and are shown at 15× magnification.

of the 12 insertions lie within three different genes, *MXAN_4710*, *MXAN_6679*, and *MXAN_7103*, previously not known to be required for S motility (Figures 1 and 2). Many *M. xanthus* strains that carry a mutation in an A motility gene and an S motility gene are completely devoid of motility (Spormann and Kaiser 1999).

When the triple mutants (MxH1189, MxH1195, and MxH1198) were examined by time-lapse videomicroscopy, subtle differences in their motility were identified. More than 80% of wild-type (DK1622) cells move during the 20 min video with a typical speed of 2.7 μm/min (range 0.8–3.2 μm/min) on CTPM agar pads. In contrast, <5% of the cells of strain MxH1104 displayed any movement at all. Those that do move, move at the much slower rate of ~0.1 μm/min. Isolated cells of MxH1104 do not show any movement. The majority of cells of strains MxH1189 and MxH1198 show no movement on CTPM agar pads, but a subset (5%) of MxH1195 cells show a rapid back-and-forth movement similar to that described by Spormann and Kaiser (1999) for *mglA* mutants.

Methylcellulose has been found to restore the motility of a subset of S mutants, because it can stimulate the retraction of type IV pili (Sun *et al.* 2000; Li *et al.* 2003). Therefore, we compared the motility of triple mutants with the motility of the wild-type and parental MxH1104 strains in the presence of 1% methylcellulose. In contrast with their movement on CTPM agar pads, most (>90%) MxH1104 cells, including isolated cells, were found to be motile in the presence of methylcellulose. Although their rate of movement (2.3 μm/min) is similar to that of the wild type, MxH1104 cells reverse direction every 1–2 min, whereas wild-type cells reverse direction approximately every 6 min. About one-third (28/80) of MxH1189 cells actively rotate in the presence of methylcellulose, whereas no MxH1189 cells show movement on a CTPM surface. The motility of MxH1195 is nearly identical in the presence of methylcellulose as it is on CTPM agar pads. Whereas MxH1198 cells are nonmotile on CTPM agar, >60% of these cells are motile in the presence of methylcellulose. However, these cells display erratic, jerky movements in contrast with the characteristic, smooth movement of wild-type or MxH1104 parent cells under the same conditions.

The fact that most of these insertions lie within genes required for S motility confirms our conclusion that the *masK*-815 suppressor restores S, but not A, motility in an *mglA*-8 genetic background. The majority of genes known to be required for S motility are required for the biogenesis of polar type IV pili, the exopolysaccharide (EPS) component of peritrichous fibrils, or lipopolysaccharide (LPS). In a previous mutant hunt for genes required for S motility, we mutagenized an A (Δ*aglU*) mutant of *M. xanthus* with *magellan-4* and defined the sites of 128 independent insertions resulting in a nonmotile phenotype in this different genetic background. We found that the majority of *magellan-4* insertions resulting in an S mutant phenotype inactivate genes in two large, contiguous regions of the *M. xanthus* genome, the *pil* and *eps* gene clusters, required for pili and EPS biogenesis, respectively. Four of the 128 insertions were found within *sglK*, only one insertion was found in the *sgmU* gene,

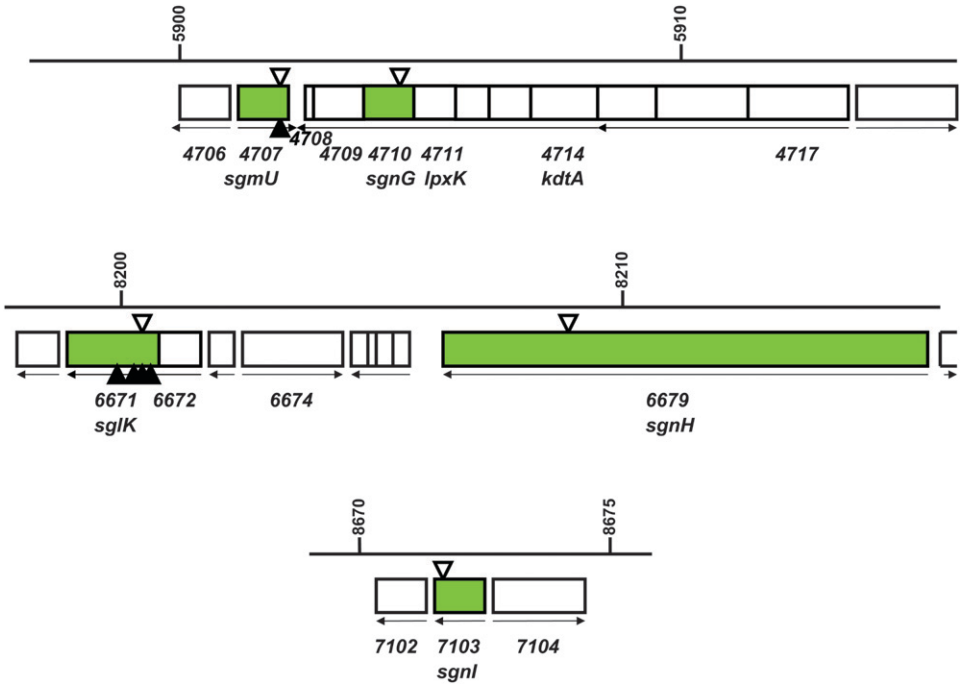

FIGURE 2.—*Magellan-4* insertions in the *mglA-8 masK-815* genetic background identify three additional genes required for S motility. The regions of the *M. xanthus* genome with the *MXAN_4710*, *MXAN_6679*, and *MXAN_7103* genes are shown. Genes are depicted as boxes, with arrows below the boxes indicating their directions of transcription. Coordinates and gene numbers are those of the *M. xanthus* genome sequence (http://cmr.tigr.org/tigr-scripts/CMR/GenomePage.cgi?org_search=&org=gmx). The positions of *magellan-4* insertions obtained in the current mutant hunt are indicated by open triangles above the genes; those of *magellan-4* insertions obtained in our previous mutant hunt (YOUDERIAN and HARTZELL 2005) as filled triangles below the genes. Adjacent genes are considered to be in the same transcription unit if they are separated by <20 bp. (Top) Genes *MXAN_4711* (*lpxK*) and *MXAN_4714* (*kdtA*), predicted to be upstream of *MXAN_4710* in the same operon, are predicted to encode essential functions required for the synthesis of the lipid A-KDO moiety of O-antigen to which heptoses are then attached. *MXAN_4710* and *MXAN_4709* together encode the two functional domains of the enzyme HldE (RfaE); *MXAN_4710* is predicted to encode D-β-D-heptose 7-phosphate kinase, and *MXAN4709* is predicted to encodes D-β-D-heptose 1-phosphate adenosyltransferase. *MXAN_4708* is predicted to encode a 55 amino acid hypothetical protein. Gene *MXAN_4717*, which may be the first gene in this operon, is predicted to encode a protein with both DnaJ and response regulator domains. (Middle) The gene *MXAN_6672* is predicted to be upstream of *sglK* (*MXAN_6671*) (WEIMER *et al.* 1998) in the same operon. It likely encodes a homolog of GrpE, which participates with DnaK in the HSP70 chaperone complex (SZABO *et al.* 1994). We have yet to obtain *magellan-4* insertions in this gene. If this gene is not essential (the *M. xanthus MXAN_4331* gene likely encodes a second homolog of GrpE), then we predict that these insertions will also result in an S motility defect, because such insertions should, at the least, be polar on the expression of *sglK*. The *sgnH* gene (MXAN_6679) appears to be the only gene in its operon. (Bottom) The *MXAN_7103* gene is predicted to encode a homolog of CheR; it also appears to be the only gene in its operon.

predicted to encode a heptosyl transferase, and only one insertion was found in the *sgnC* gene, predicted to encode one of six different response regulators required for S motility (YOUDERIAN and HARTZELL 2005).

Half (6/12) of the insertions resulting from our current mutant hunt lie within the *pil* and *eps* gene clusters, and 3 of the 12 insertions lie within the *sglK*, *sgmU*, and *sgnC* genes. The fact that we obtained independent insertions in our current hunt in the *sgmU* and *sgnC* genes, predicted to be the only genes in their transcription units (Figure 2), confirms that these genes are required for S motility. Among the 12 independent *magellan-4* insertions that disrupt S genes, only 4 of these are found at the same sites in the genes we identified in our previous mutant hunt. This result shows that the spectrum of *magellan-4* insertions that we have obtained in the *mglA masK* double mutant background is very different than the spectrum we obtained in the Δ*aglU* genetic background.

The addition of heptose to the lipid A-KDO component of LPS is required for S motility: Three of the *magellan-4* insertions define new S motility genes. One of these is in *MXAN_4710*, which appears to be the third to last gene in a large operon of at least seven genes predicted to encode proteins involved in the biosynthesis of the lipid A, KDO (3-deoxy-D-manno-octulosonic acid), and additional inner core components of LPS (Figure 2). The *MXAN_4710* gene, identified by the *mis-198* allele, is predicted to encode a protein with the N-terminal domain of the bifunctional enzyme RfaE, required for the synthesis of D-glycero-D-manno-heptose-1-phosphate. The C-terminal domain of RfaE required for the subsequent transfer of this substrate to ADP to form ADP-D-glycero-D-manno-heptose is predicted to be encoded by *MXAN_4709*, immediately downstream of *MXAN_4710*. It may be the case that this insertion not only inactivates *MXAN_4710*, but also is polar on the expression of *MXAN_4709*. The nearby convergently transcribed *MXAN_4707* gene is predicted to encode RfaF, ADP-heptose:LPS heptosyltransferase, which uses the product of the reaction catalyzed by RfaE to add heptose to initiate the formation of the inner core of LPS. The result that we obtain insertions in both *MXAN_4710* and *MXAN_4707* and the fact that *MXAN_4710*, *MXAN_4709*, and *MXAN_4707* are the only three genes present in the *M. xanthus* genome sequence predicted to encode

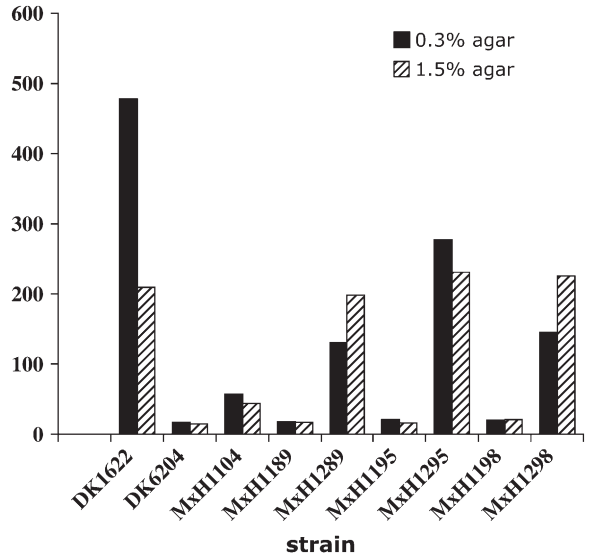

FIGURE 3.—*Magellan-4* insertions in the *MXAN_4710 (sgnG), MXAN_6679* (*sgnH*), and *MXAN_7103* (*sgnI*) genes result in defects in S motility but not A motility. The spreading areas of single (MxH1289, MxH1295, and MxH1298), double (DK6204 and MxH1104), and triple mutants (MxH1189, MxH1195, and MxH1198) are compared with the spreading of the wild-type strain (DK1622) on CTPM medium containing 0.3 and 1.5% agar. The final spreading area is the difference between the colony area at $T = 0$ and $T = 120$ hr after incubation at 32°.

activities required for the addition of heptose to LPS, argue that the addition of heptose to the inner core of LPS is required for S motility.

These results confirm the finding that the biosynthesis of O-antigen is required for S motility (BOWDEN and KAPLAN 1998; YANG *et al.* 2000a,b; YOUDERIAN and HARTZELL 2005). We do not as yet know whether LPS plays a direct role in the mechanism of S motility by participating in cell–cell recognition, or an indirect role in S motility, because mutations that result in defects in LPS biosynthesis have pleiotropic effects on the biogenesis of EPS, as well as on the proportionation of reduced carbon into biopolymers, since more than half of the reduced carbon in many Gram-negative bacteria is invested into O-antigen.

Using generalized transduction with phage Mx8, we were unable to backcross the *mis-198* allele into a wild-type genetic background to confirm its role in S motility. This is because mutants with this mutation, like other "rough" mutants defective in O-antigen biosynthesis, are resistant to phage Mx8, which requires O-antigen for adsorption (FINK *et al.* 1989). Therefore, to determine if disruption of *mis-198* affects S motility in a wild-type genetic background, chromosomal DNA from *M. xanthus* MxH1198 was transferred into *M. xanthus* DK1622 by electroporation. As shown in Figure 3, MxH1298 (which carries the *sgnG*::*magellan-4* insertion *mis-198*, in an otherwise wild-type genetic background) displays reduced spreading on 0.3% CTPM agar compared with that of the wild-type strain, but shows a near normal level of spreading on 1.5% agar, characteristic of mutants defective in S motility. As shown in Figure 4, isolated cells are present at the edges of colonies formed by the MxH1298 mutant strain.

A homolog of the CheR methyltransferase is required for S motility: The second new S gene *MXAN_7103* is predicted to encode a homolog of CheR methyltransferase and appears to be expressed in its transcription unit as a single gene (Figure 2). *M. xanthus* is predicted to encode nine paralogs of CheR, one of which is encoded by the *frzF* gene. FrzF methyltransferase is required for the sensory adaptation of *M. xanthus* in response to repellent chemotactic stimuli (SHI and ZUSMAN 1993), and the multicellular development of *M. xanthus* at lower cell densities (KASHEFI and HARTZELL 1995). Although FrzF participates in the Frz chemotaxis system of *M. xanthus*, it is not required for social motility.

To confirm that the *mis-189* insertion confers an S motility defect, we backcrossed this *magellan-4* insertion into a wild-type genetic background by electroporation to produce *M. xanthus* MxH1289. All Kan^R electroporants (34/34) formed smaller colonies than their wild-type (DK1622) parent. As shown in Figures 3 and 4, the MxH1289 mutant is defective in spreading on 0.3% agar. MxH1289 is delayed in fruiting body formation and forms mounds after 4 days whereas the wild-type forms mounds within one day on TPM starvation agar (P. L. HARTZELL, unpublished data). The production of heat-resistant spores is reduced 10-fold compared with the wild type.

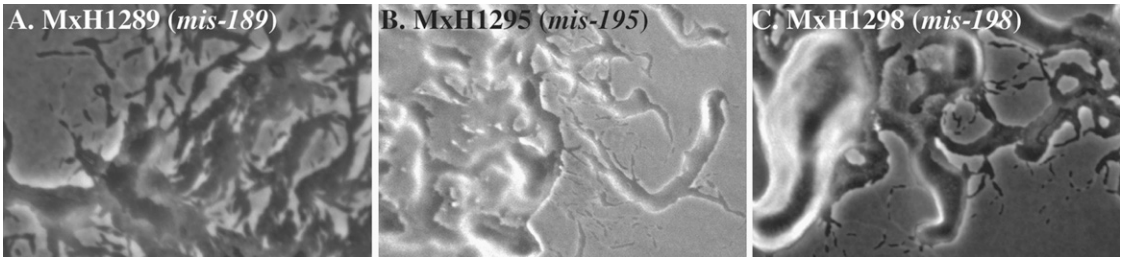

FIGURE 4.—Gliding motility of isolated cells is evident in the *MXAN_4710 (sgnG), MXAN_6679* (*sgnH*), and *MXAN_7103* (*sgnI*) mutants. The colony edge morphology of *M. xanthus* strains (A) MxH1289, (B) MxH1295, and (C) MxH1298 are shown. The 15× (final magnification) images were taken with a Nikon FXA microscope.

The result that *MXAN_7103*, identified by the *mis-189* allele, is essential for S motility suggests that a methyl-accepting chemotaxis protein must also play an essential role in S motility. This substrate of the *MXAN_7103*-encoded methyltransferase may be the product of the *difA* gene, predicted to encode a methyl-accepting chemotaxis protein, which is required for the production of extracellular fibrils, and therefore for S motility (Yang *et al.* 1998). However, because there is no evidence that methylation of DifA occurs (Bonner *et al.* 2005), the product of *MXAN_7103* may participate in a different chemotaxis pathway required for S motility. We are currently testing whether a mutation in this gene affects EPS production. Although the *dif* gene cluster encodes other homologs of chemotaxis proteins as well as DifA, it does not include a gene encoding a paralog of CheR (Yang *et al.* 1998). *Pseudomonas aeruginosa* also has cluster of chemotaxis genes required for S (or "twitching" motility). This cluster includes a gene encoding a required methyl-accepting chemotaxis protein, but the gene encoding its partner methyltransferase is not linked with this cluster (Kato *et al.* 1999).

The *MXAN_6679* gene, predicted to encode a large YD repeat protein, is required for S motility: The third new S gene *MXAN_6679* also appears to be expressed in its transcription unit as a single gene. It is predicted to encode a product with a N-terminal signal sequence, which is cleaved after residue 30, to yield a protein with a predicted molecular mass of ~340,000 Da. The 3146 amino acid protein product of *MXAN_6679* has an unusual domain structure that includes at least 30 repeats that are typically 21 amino acids in length and include the dipeptide Tyr–Asp (YD, Figure 5). These repeats are found in very few proteins, including the predicted protein products of the *rhs* (rearrangement hot spot) elements of *E. coli* (Hill *et al.* 1994), the cell-wall associated WapA protein of *Bacillus subtilis* (Foster 1993), toxin A of *Clostridium difficile* (Dove *et al.* 1990), the first pair-rule protein Ten-m of *Drosophila melanogaster* (Baumgartner *et al.* 1994; Levine *et al.* 1994), and the vertebrate teneurin-1 protein (Minet *et al.* 1999). The four latter proteins are secreted extracellular proteins; among these, the vertebrate protein is critical for neuronal development (Minet *et al.* 1999). Although little is known about the function of the YD repeat motifs in WapA and Ten-m, the YD repeat motifs of both *C. difficile* toxin A and of teneurin-1 have been shown to bind carbohydrate (Krivan *et al.* 1986; Wren 1991; Minet *et al.* 1999), and the *E. coli* RhsA protein has been shown to be involved in the export of EPS (Mcnulty *et al.* 2006).

To confirm that the *mis-195* insertion in *MXAN_6679* confers an S motility defect, we backcrossed this *magellan-4* insertion into a wild-type genetic background to produce *M. xanthus* MxH1295. The Kan^R determinant in MxH1195 (*mglA-8 masK-815 mis-195*) is 100% linked to a defect in S motility. All Kan^R electroporants form smaller colonies than their wild-type parent (DK1622) that are

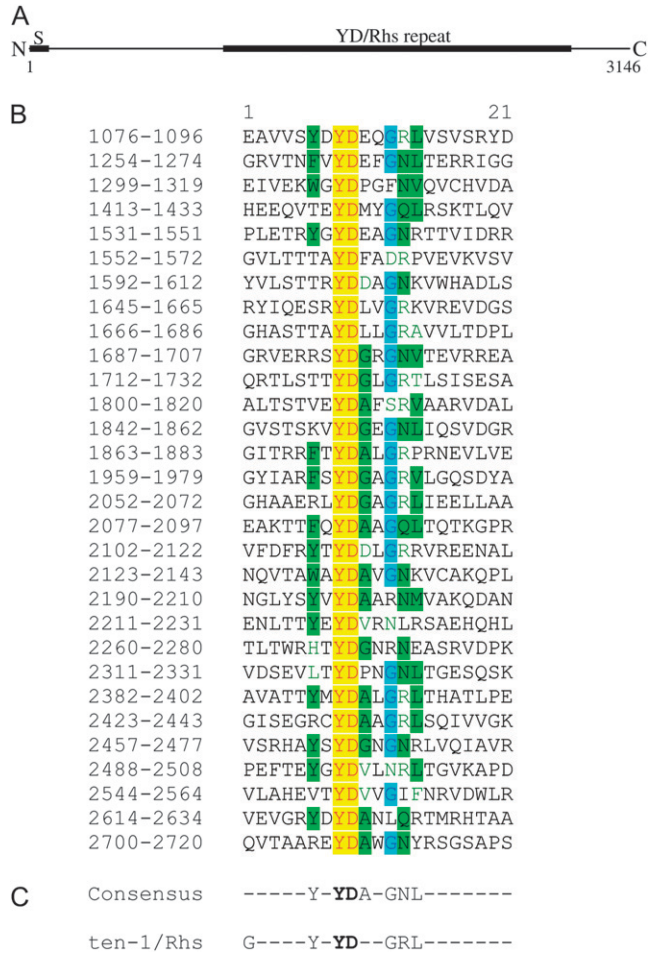

Figure 5.—*MXAN_6679* is predicted to encode a protein product with 36 YD repeats. The product of *MXAN_6679 (sgnH)* is predicted to be a 340-kDa protein. The presence of an N-terminal signal sequence, shown in A, suggests that SgnH is secreted. Over half of the protein includes a series of YD/Rhs repeats (solid bar from 1076 to 2720). Rhs proteins contain extended repeat regions that are thought to be involved in ligand binding. The extracellular repeat contains the dipeptide YD. Potential YD repeats in the predicted product of the *MXAN_6679* (*sgnH*) gene were aligned by visual inspection and by SMART (Simple Modular Architecture Research Tool) Letunic *et al.* 2002. Repeat regions, shown in B, are numbered by the residue numbers of the predicted protein product of the *sgnH* gene. The consensus sequence of the YD repeats shared by the vertebrate teneurin-1 protein and the predicted products of the *E. coli rhs* genes (Minet *et al.* 1999) is shown in C. Amino acids occurring in the consensus sequence are shaded.

defective in spreading on 0.3% agar (Figure 3). Their ability to spread on 1.5% agar is similar to that of the wild type. Isolated cells are detected at the edge of vegetative colonies formed by MxH1295, characteristic of A motility (Figure 4). MxH1295 is able to produce fruiting bodies that are similar in size and color with those of the wild type, but the production of heat-resistant spores in these fruit is reduced 90-fold (P. L. Hartzell, unpublished data).

What role might this YD repeat protein play in the mechanism of S motility of *M. xanthus*? In *D. melanogaster*, the Ten-m protein was identified using an antibody specific for phosphotyrosine (Levine *et al.* 1994). Thus, YD repeats may represent targets for tyrosine phosphorylation, perhaps by the essential MasK tyrosine kinase of *M. xanthus* (Thomasson *et al.* 2002). If the product of *MXAN_6679* is secreted, as we predict, then it may play a role for sensing the presence of the carbohydrate polymers that are a component of EPS. Additional biochemical studies of this unusual protein product, and a more careful analysis of its roles in S motility and multicellular development, may help us understand why the Rhs elements, predicted to encode similar YD repeat proteins, have been acquired recently by the Gram-negative enteric pathogens (Hill *et al.* 1994).

DISCUSSION

When we compare the results of a previous mutagenesis to obtain *magellan-4* insertions in S genes starting with an A mutant genetic background, we find that the spectrum of *magellan-4* insertions in S motility genes is different from that when we screen for nonmotile mutants starting with the *mglA*-8 *masK*-815 genetic background. Although many of the insertions we obtained in the latter double mutant background are in the same genes "hit" with 128 independent insertions starting with an A mutant, 3 of 12 are not. This is not surprising. The differences in genetic background may favor the recovery of a different subset of mutations in S motility genes. Such differences also help to distinguish components that function in the signaling pathways needed for S motility from structural components. We did not recover *magellan-4* insertions in *mglA* or *masK* although disruption of either gene in the *M. xanthus* MxH1104 background would be expected to yield nonmotile colonies. This result is consistent with our previous results showing that *masK* is an essential gene. Disruption of *mglA* in a wild-type background does not affect cell growth and hence *mglA* is not essential. However the pairing of the *mglA8* mutation with the *masK*-815 suppressor of *mglA* might make it difficult to identify strains with insertions in *mglA*.

We find that a mutation in *MXAN_4707* (*sgnG*; *M. xanthus* MxH1198), which is predicted to encode an enzyme required for addition of heptose to the inner core of LPS, abolishes motility on CTPM agar. However, the fact that methylcellulose stimulates the movement (albeit jerky) of this mutant suggests that its type IV pili are capable of retraction. Hence, SgnG may play a role in the signaling pathway that stimulates the retraction of type IV pili, required for S motility. The motility of cells carrying a disruption of the *sgnH* gene (*MXAN_6679*), predicted to encode a 340-kDa extracellular YD-repeat protein, is not stimulated by the addition of methylcellulose. This result suggests that SgnH may be a structural component for the S motor. Finally, cells carrying a disruption of the *sgnI* gene (*MXAN_7103*), predicted to encode a protein with methyltransferase activity, exhibit rotational movement that occurs only in the presence of methylcellulose. We speculate that this rotational movement indicates that the mutant can produce and retract type IV pili, yet is blocked in the ability to switch the extension of pili from one cell pole to the other. Hence, these cells, which adhere by their polar pili to the surface of a microscope slide, can retract their pili when methylcellulose is present. If SgnI is involved in polar switching, it may be an integral part of the Mgl signal transduction pathway that coordinates reversals of the A and S motors. Specifically, SgnI may an S-gliding specific component that is downstream of the Mgl coordination signal, so a mutation in *sgnI* would not be expected to affect A gliding.

The 140 *magellan-4* insertions, which we have characterized in a recent and in this current study, identify 36 new genes required for S motility; when added to the 77 genes characterized previously, we now know the identities of >113 different genes required for S motility. The result that 3 among 12 independent insertions of *magellan-4* in an *mglA-8 masK-815* genetic background that impair motility identify three new genes involved in S motility argues that we have not saturated the genes required for S motility. This idea is supported by the fact that we have found *magellan-4* insertions in only three-quarters of the genes shown to be involved in S motility by all genetic methods employed to date. The mechanism of S motility is extraordinarily complex and will likely involve two to four times the number of genes required for the mechanism of flagellar-dependent motility in the Gram-negative enteric bacteria.

The choice of different starting genetic backgrounds for screening S mutants made by transposon insertions will likely improve our chances of finding additional S motility genes. Thus, we expect that the characterization of additional *magellan-4* insertions in the double mutant *mglA masK* genetic background will reveal additional genes required for S motility. Second, because many S genes are clustered in the genome sequence, detailed analyses of mutations in nearby genes likely will identify additional genes required for S motility. This has certainly proven to be the case for the genes required for the biogenesis of EPS. For example, the result that both the *sglK* (MXAN_6671) and *sgnH* (MXAN_6679) genes are required for S motility suggests that the seven genes between these two also may be required for S motility. Third, because many of the genes known to be involved in S motility are predicted to encode products of related function, genome-based approaches involving the systematic inactivation of genes predicted to encode functions similar to those of known S motility genes will certainly complement our more classical genetic methods, including transposon mutagenesis, to identify additional S motility genes.

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