

New Locus Important for *Myxococcus* Social Motility and Development[∇]

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Received 14 June 2007/Accepted 20 August 2007

The *mts* locus in salt-tolerant *Myxococcus fulvus* HW-1 was found to be critical for gliding motility, fruiting-body formation, and sporulation. The homologous genes in *Myxococcus xanthus* are also important for social motility and fruiting-body development. The *mts* genes were determined to be involved in cell-cell cohesion in both myxobacterial species.

Myxobacteria are gram-negative gliding bacteria with a multicellular developmental life cycle (20, 23). Gliding motility in these cells plays a crucial role in myxobacterial predatory and developmental aggregation (23). There are two distinct motility systems controlling *Myxococcus* gliding: the adventurous (A) system, which controls the movement of individual and isolated cells, and the social (S) system, which is essential for the swarming and aggregation of cells (7, 8). With the dual motility system, myxobacteria are able to adapt to different environments, such as dry and highly hydrated agar surfaces.

Although most myxobacteria are found in soil, there are salt-tolerant species from marine environments (5, 9–11, 17, 28). In contrast to the salt sensitivity of terrestrial strains (19), salt-tolerant myxobacteria are able to withstand a wide range of salinity. In response to changes in salinity, some salt-tolerant myxobacteria show altered vegetative and developmental characteristics (29). Differences in morphogenetic characteristics appear to indicate that salt-tolerant myxobacteria are the result of the adaptation of soil myxobacteria to marine environments (29). Analysis of salt-tolerant *Myxococcus* strains has revealed that they may have retained dual gliding motility systems. Some of the high-salt-tolerant strains exhibited enhanced S motility in the presence of seawater, as indicated by increased swarming on soft agar (24).

To identify the S motility genes in the salt-tolerant *Myxococcus fulvus* strain HW-1, a genetic screen was performed using transposon MiniHimar1-*lacZ* electroporation (14), which yielded a transformation efficiency of 10 to 10² CFU/μg DNA. Of more than 2,000 insertion mutants, 21 were deficient in motility and formed small colonies compared to those of the wild-type parent strain HW-1. One of the mutants showed a significant reduction in colony expansion on a 0.3% agar surface, which is indicative of an S motility defect. The mutated gene led to the discovery of the *mts* locus, which is the focus of this report.

Phenotypic characteristics of the mutant HL-1. The mutant HL-1 (Table 1) was assessed for motility phenotypes by

standard methods (21). As shown in Fig. 1A to D, the swarming colony sizes of HL-1 were 90.4% and 84.2% smaller than those of HW-1 on hard and soft agar, respectively. On soft agar, the mutant produced small colonies with a rough, dentate swarm edge (Fig. 1C), in contrast to the large colonies with the translucent smooth lacy swarm edge of the wild-type strain (Fig. 1D). At their swarming edges on hard agar, HL-1 cells moved mainly as individuals, with a few in small groups (Fig. 1E), whereas HW-1 cells translocated over the agar surfaces either as individuals or in groups (Fig. 1F). The phenotypes of the mutants mimic those of some *Myxococcus xanthus* A⁺S⁻ mutants deficient in extracellular polysaccharides (EPS), such as DK3468 (*dsp*) (22) and YZ603 (*ΔdifE*) (2), suggesting that the mutant HL-1 is defective in social motility.

The mutant HL-1 was assessed for developmental ability on TPM starvation medium by methods described previously (15). The mutant cells formed a weak and rudimentary fruiting-body structure (Fig. 1G), compared to the mature fruiting bodies of wild-type cells (Fig. 1H). The sporulation frequency of HL-1 was only 0.67% that of HW-1, which was able to develop about 3.0 × 10⁶ spores from an initial input of 5 × 10⁷ cells after a 5-day incubation. These results indicate that the mutant HL-1 is also significantly defective in developmental aggregation and sporulation.

Cells of the mutant HL-1 dispersed easily in liquid culture, indicating possible defects in cell cohesion. The amount of EPS was assessed by scanning electron microscopy, which revealed less extracellular matrix on the surfaces of HL-1 cells than on those of HW-1 cells. The dyes Congo red and trypan blue, which bind to EPS (3, 26), were employed for quantitative analysis of the extracellular matrices of the wild-type and mutant strains by the method described previously (2). The wild type strain HW-1 bound 69.8% and 48.4% of Congo red and trypan blue, respectively, compared to 49.8% and 22.9% for HL-1, indicating less cohesion ability of the mutant. A clumping assay, using the method described previously by Shimkets (22), also confirmed that the mutant cells exhibited less cohesion than the wild-type cells, and the relative absorbance readings at the 100-min end point for the mutant and wild-type cells in morpholinepropanesulfonic acid (MOPS) buffer were 0.563 ±

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[∇] Published ahead of print on 24 August 2007.

TABLE 1. Myxobacterial strains and plasmids

Strain or plasmid	Relevant characteristic	Reference or source ^a
Strains		
<i>M. fulvus</i>		
HW-1 (ATCC BAA-855)	Wild type	17
HL-1	<i>mtsC</i> ::MiniHimar1- <i>lacZ</i>	This study
<i>M. xanthus</i>		
DK1622	Wild type	13; D. Kaiser, Stanford University
DK1217	<i>aglB1</i>	8; Z. M. Yang, Virginia Tech
DK10410	Δ <i>pilA</i>	25; M. H. Singer, University of California, Davis
ZC16-4	Δ MXAN1334	This study
ZC12-3	<i>aglB1</i> Δ MXAN1334	This study
ZC10-1	Δ <i>pilA</i> Δ MXAN1334	This study
ZC16-23	Deletion of MXAN1332 to MXAN1337	This study
Plasmids		
pMiniHimar1- <i>lacZ</i>	Km ^r <i>lacZ</i>	H. B. Kaplan, University of Texas
pBJ113	Gene replacement vector with KG cassette; Km ^r	12; Z. M. Yang, Virginia Tech
pZCY6	MXAN1334 in-frame deletion in pBJ113	This study
pZCY9	MXAN1332 to MXAN1337 deletion in pBJ113	This study

^a Virginia Tech, Virginia Polytechnic Institute and State University.

0.086 and 0.075 ± 0.055 optical density units at 600 nm, respectively.

We reported previously that the salt-tolerant *Myxococcus* strains exhibited enhanced S motility in the presence of seawater on either soft or hard CYE agar (24). Interestingly, the effect of seawater on swarming ability was significantly decreased by the mutation (Fig. 2). These results suggest that the mutated gene(s) is involved in or responsible for the enhance-

ment of surface translocation in response to the presence of seawater.

The mutagenized gene in HL-1 and the related genes in this locus. The MiniHimar1-*lacZ* transposon contains the *Escherichia coli* replication origin R6K. To identify the gene mutated in HL-1, its genomic DNA was digested with SphI and BamHI, self-ligated for *E. coli* transformation, and then sequenced. Two thermal asymmetric interlaced PCR amplifications (18)

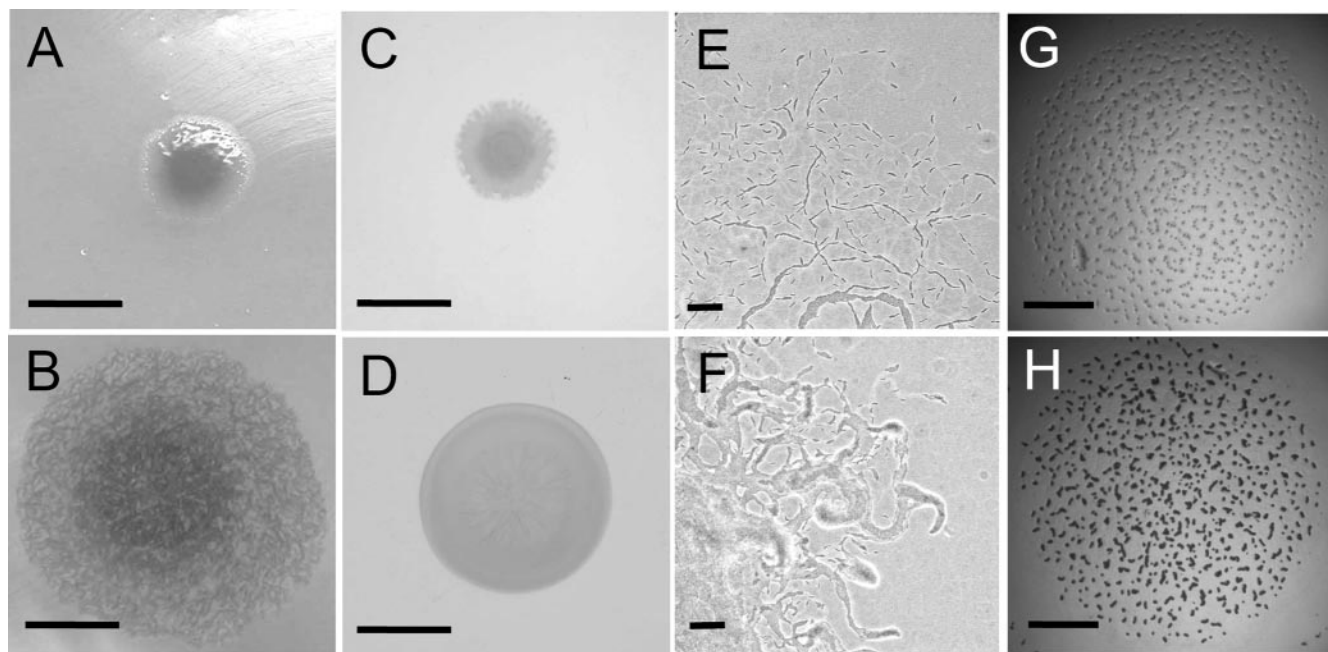


FIG. 1. Gliding behavior and fruiting-body development of the *M. fulvus* wild-type strain HW-1 (B, D, F, and H) and its mutant HL-1 (A, C, E, and G). Colony expansions were done on CTT medium with 1.5% (A and B) or 0.3% (C and D) agar for 3 days. Colony edge morphologies were done on CTT hard (1.5%) agar (E and F). Development of fruiting bodies was carried out on TPM plates (G and H) for 5 days, with inoculation of 5×10^9 cells/ml. The plates were incubated at 30°C. Bars, 0.6 cm for panels A to D, 30 μ m for panels E and F, and 1.5 mm for panels G and H.

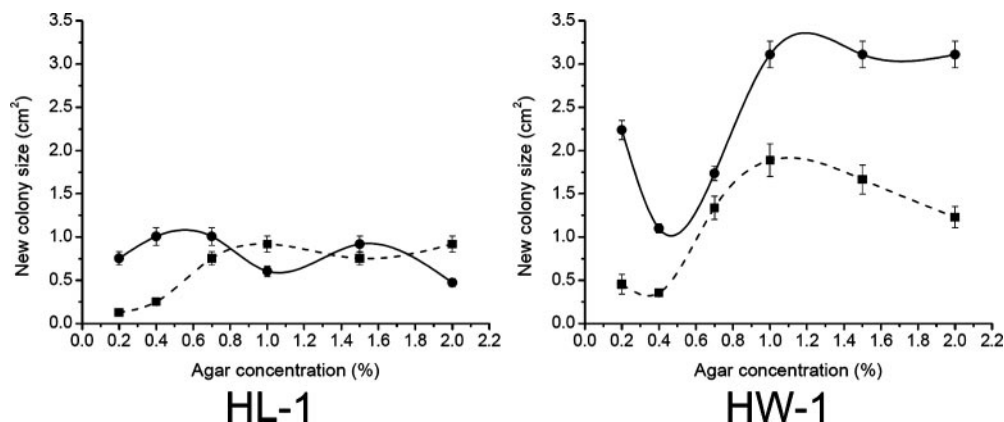


FIG. 2. Swarming colony sizes of the mutant HL-1 and the wild-type strain HW-1 on the nutrient medium CTT without (dashed lines) or with (solid lines) 20% seawater and with different concentrations of agar.

were then performed. The nested specific primers and arbitrary degenerate primers (AD primers) used in this study are listed in Table 2. An upstream 6.3-kb segment and a downstream 6.7-kb segment flanking the insertion were obtained. After sequencing, the junction sequence between the two segments was further PCR amplified from the wild-type strain HW-1 and sequenced. By using the FramePlot program, version 2.3.2 (<http://www.nih.go.jp/~7Ejun/cgi-bin/frameplot.pl>), the 13-kb segment (deposited in GenBank with the accession number EF371498) was predicted to contain six open reading frames (ORFs), which likely form a gene cluster (Fig. 3). Blast against the GenBank database revealed that the sequences are significantly homologous to the corresponding ORFs from *M. xanthus* DK1622 and *Stigmatella aurantiaca* DW4/3-1. Most of these ORFs are predicted to be putative type 3 thrombospondin genes. Type 3 thrombospondin, reported for eukaryotic cells, has affinity for cell surfaces, calcium ions, and many matrix macromolecules (1, 4, 16). Thus, the gene cluster was designated *mts*, for myxobacterial thrombospondin-like proteins (MtsA to MtsF). The MiniHimar1-*lacZ* insertion interrupted the codon for Tyr359, located 83 residues from the C terminus of the predicted MtsC protein. MtsA and MtsE possess transmembrane regions at their N termini, as assessed by SMART (<http://smart.embl-heidelberg.de/>). The SignalP-HMM program (<http://www.cbs.dtu.dk/services/SignalP/>) predicted that MtsA, MtsC, MtsD, MtsE, and MtsF contain signal peptides with probabilities of 0.998, 1.000, 0.997, 0.984, and 0.997

TABLE 2. Primers used for TAIL-PCR amplification^a

Primer	Length (bp)	Sequence (5' to 3')	T_m (°C)
Specific primers			
1332-LTL1	25	TCCGGCTGATAGCGATGCGCCACCC	70.2
1332-LTL2	25	GCATCCACCACCGTCAAGGGAAGTC	66.9
1332-LTL3	25	CGGCTGGGATGTTTCATTGCTCTTG	63.6
TL-1	25	CAGCGGGCAGTTGTCTACATCGTTG	65.3
TL-2	21	TTGGAGACGAA GGGGCAGTTG	61.9
TL-3	23	TGTCGCCGTCGTCCTGTAGTTC	65.5
AD primers			
AD4	16	TG(A/T)GNAG(A/T)ANCA(G/C)AGA	43.9
AD7	16	CA(A/T)CGICNGAIA(G/C)GAA	43.0

^a TAIL-PCR, thermal asymmetric interlaced PCR; T_m , melting temperature.

and that the potential cleavage sites are at residues 24, 25, 21, 20, and 22, respectively. Bioinformatics analysis of the predicted *mts* gene, together with the phenotypes of the mutant, suggested that the Mts proteins are probably involved in the construction of the cell surface matrix for S motility and development.

Characteristics of *M. xanthus* *mts* mutants. Genetic manipulation of HW-1 is difficult. Attempts to make targeted *mts* mutations in HW-1 using established protocols for *M. xanthus* (12) were unsuccessful (data not shown). Instead, an in-frame deletion of *mtsC* (429 amino acid residues were deleted from 496 amino acids of MXAN1334) was performed for *M. xanthus* strains DK1622 ($A^+ S^+$), DK1217 ($A^- S^+$), and DK10410 ($A^+ S^-$) to determine the function of *mtsC*. The mutants in the $A^+ S^+$ and $A^- S^+$ backgrounds formed smaller colonies on soft agar than their parent strains, whereas the mutation in the $A^+ S^-$ background did not lead to changes in the colonies on either hard or soft agar (Fig. 4). The results indicated that *mtsC* is likely also involved in S motility in *M. xanthus*.

Interestingly, the effect of the in-frame deletion on motility in *M. xanthus* seems to be less marked than that of the insertion in *mtsC* in *M. fulvus* HW-1. To determine whether the Mts proteins are important for S motility and development in *M. xanthus*, we completely deleted the sequence of MXAN1332 to MXAN1337 from DK1622. Plasmid pZCY9, which contains a deletion of all the *mts* genes, was transformed into DK1622 to give rise to the mutant ZC16-23. ZC16-23 produced smaller colonies (about 75% the size of colonies of the wild-type strain DK1622) on hard CTT (1% (wt/vol) Casitone, 8 mM $MgSO_4$, 1 mM K_2HPO_4 - KH_2PO_4 [pH 7.6], and 10 mM Tris-HCl [pH 7.6]) agar (Fig. 4). Compared to the change of the colony size

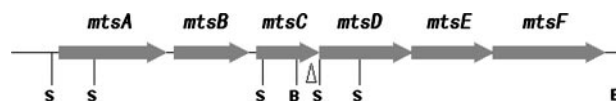


FIG. 3. Physical organization of the *mts* region (13.0 kb) on the *M. fulvus* chromosome and the MiniHimar1-*lacZ* insertion site (indicated by an open arrowhead). The ORFs and predicted transcription directions are represented by arrows. The locations of the restriction enzyme sites used for the cloning sequence are shown. S, SphI; B, BamHI.

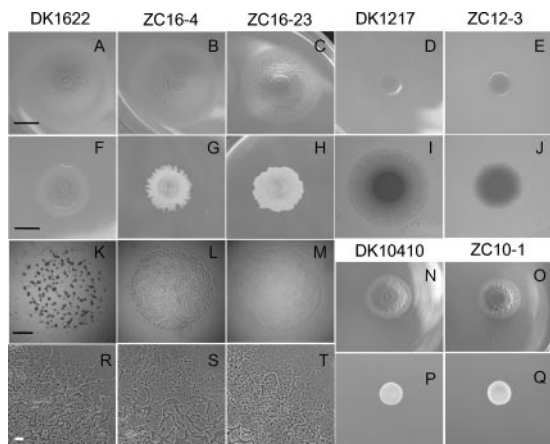


FIG. 4. Morphological characteristics of the wild-type strain *M. xanthus* DK1622 and its mutants ZC16-4 (Δ MXAN1334) and ZC16-23 (deletion of MXAN1332 to MXAN1337); the $A^- S^+$ strain *M. xanthus* DK1217 and its mutant ZC12-3 (Δ MXAN1334); and the $A^+ S^-$ strain *M. xanthus* DK10410 and its mutant ZC10-1 (Δ MXAN1334). Colony expansions were done on CTT medium with 1.5% (A to E, N, and O) (bar, 1.0 cm) and 0.3% (F to J, P, and Q) (bar, 0.7 cm) agar for 5 days. Development of fruiting bodies was done on TPM plates (K to M) (bar, 1.5 cm) for 5 days. The plates were incubated at 30°C. (R to T) Cell movement at the swarming edges of DK1622, ZC16-4, and ZC16-23 on 1.5% agar. Bar, 50 μ m.

in *M. fulvus* HL-1 by the insertion in *mtsC*, the effect of the complete deletion of *mts* in *M. xanthus* is not as prominent. The MXAN1334 insertion mutant exhibited motility phenotypes similar to those of the deletion mutant ZC16-4 (data not shown). These results indicate that the motility differences between the *M. fulvus* HW-1 insertion mutant and the *M. xanthus* DK1622 deletion mutant were not likely due to polar effects of transposon insertion. The *mts* products obviously play a more important role in S motility in *M. fulvus* HW-1 than they do in *M. xanthus* DK1622.

In contrast to their role in S motility, Mts proteins are essential for fruiting-body formation and sporulation in *M. xanthus*. When inoculated onto TPM (10 mM Tris HCl [pH 7.6], 8 mM $MgSO_4$, 1 mM $K_2HPO_4 \cdot KH_2PO_4$ [pH 7.6], and 1.5% agar) plates, DK1622 cells formed visible fruiting-body structures from the second day of incubation; whereas the mutants ZC16-4 and ZC16-23 did not form fruiting bodies even after 5 days of incubation (Fig. 4). Under our assay conditions, DK1622 cells produced 1.3×10^6 spores from an initial input of 5×10^7 cells after 5 days of incubation. The sporulation frequency of ZC16-4 cells was only 0.01% that of DK1622 cells, and no spores were detected for ZC16-23. The effects of *mts* genes on development are rather similar in *M. fulvus* HW-1 and *M. xanthus* DK1622.

Concluding remarks. S motility is a cell-cell contact-dependent mode of movement that is essential for myxobacterial predation and development. Youderian and Hartzell recently suggested that at least 25% of the nonessential genes involved in S motility had not yet been identified, probably due to preferential mutation hot spots (27). Different *Myxococcus* species or strains may possess genotypes with subtle differences in motility. The diversification of the motility genotypes thus provides a useful source and also an efficient approach to

discovering the hypomorphic motility genes in the model strain *M. xanthus* DK1622. This paper describes a new genetic locus (*mts*) that is required for S motility and development in *Myxococcus*. The *mts* locus is predicted to contain six ORFs. Four components—MtsB, MtsC, MtsD, and MtsE—were predicted to be homologous to the type 3 thrombospondins (Goldman et al. [6] predicted two, MtsC and MtsE), which are multifunctional proteins with affinity for cell surfaces, calcium ions, and many matrix macromolecules (16). The thrombospondins have been reported previously only in eukaryotes. In this paper we determined that the *mts* locus is also involved in *Myxococcus* cellular cohesion. Although the *mts* products are required in development, they affect S motility to different extents in different *Myxococcus* strains. The Mts proteins probably function cooperatively, serving as components for intercellular cohesion, like the thrombospondins in eukaryotic cells (16). However, the predicted myxobacterial thrombospondin-like proteins are similar to thrombospondin 3 only in the variable region, not in the highly conserved region or the calcium binding motifs. The structure and function of Mts are under further investigation.

We thank Dale Kaiser, Heidi B. Kaplan, Mitchell H. Singer, and Zhaomin Yang for sharing strains and plasmids; Roberta Greenwood for help in editing the manuscript; and Ed Yves V. Brun and anonymous reviewers for helpful comments on the manuscript.

This work was financially supported by grants 30270023, 30400009, and 30600007 from the Chinese National Natural Science Foundation and Chinese 863 Projects.

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