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Identification of a Gene Required for Gliding Motility in *Myxococcus xanthus*

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Abstract A novel gene (*agiA*) required for adventurous gliding motility in *Myxococcus xanthus* has been identified. Null mutations in this gene caused defects in the gliding movement of isolated cells, suggesting that it belongs to one of the A-motility genes. The isolated *agiA* mutant cells neither glided nor produced slime trails on agar surface. However, *agiA* was different from other known A-motility genes in that the *agiA* mutant created in the S^- mutant background glided in the swarm of cells, since other known A-motility mutants created in the S^- mutant background do not move in the swarm of cells. The *agiA* mutant was also defective in fruiting body development. Sequence analysis predicted that *agiA* encodes a 787-amino-acid protein with eight tripeptide repeat motifs.

Key words: Myxobacteria, gliding motility, *Myxococcus xanthus*

Gliding motility is the movement of nonflagellated cells in the direction of its long axis on a solid surface and is found in many phylogenetically diverse bacteria [21, 22, 30]. *Myxococcus xanthus*, a Gram-negative soil bacterium that is known to form multicellular fruiting bodies, has been known to possess two genetically separable gliding motility systems: adventurous(A)-motility and social(S)-motility [8, 10]. The A-motility system functions whenever cells are in a group or not, whereas the S-motility system functions only when cells are in a group. The driving force for S-motility is known to be generated by pulling cell bodies with their polar type IV pili [13], and normal S-motility also requires exopolysaccharide components of the extracellular matrix, previously termed fibrils [1, 19] and the lipopolysaccharide O antigen [3] in addition to the type IV pili. On the other hand, the mechanism of A-gliding motility is still obscure, although several models have been proposed to explain the gliding mechanism of A-motility [4, 14, 16, 20, 24, 30]. More than 34 genes required for A-motility have been identified, but their functions are not yet elucidated [25, 31, 34, 37]. Gliding myxobacteria leave trails of slime behind them, which serve as preferential gliding pathways for other cells [4, 5]. Slime primarily consists of polysaccharide [32], and lack of slime production correlates with loss of gliding motility. Recently, nozzle-like structures that appear to extrude slime have been discovered in cyanobacteria and myxobacteria, and it has been suggested that the direct slime extrusion through the nozzle might provide the driving force for bacterial gliding, including A-motility of *M. xanthus* [11, 35]. We report here the identification of a novel gene that is required for A-motility in *M. xanthus*. The cells carrying a null mutation in this gene were unable to individually move apart from other cells like other A-motility mutants. However, they could move in a swarm of cells, which makes this mutant different from other A-motility mutants.

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Materials and Methods

Bacterial Strains, Plasmids, and Culture Conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *M. xanthus* DZ4467 and DK1217 were kindly provided by David R. Zusman at UC-Berkeley. *M. xanthus* was cultured vegetatively in CYE medium, which contains 1% (w/v) casitone, 0.5% yeast extract, 10 mM morpholinepropanesulfonic acid (MOPS, pH 7.6), and 8 mM $MgSO_4$ [23]. Fruiting body development was carried out on a CF medium containing 10 mM MOPS (pH 7.6), 0.015% casitone, 8 mM $MgSO_4$, 1 mM KH_2PO_4, 0.2% sodium citrate, 0.02% $(NH_4)_2SO_4$, and 0.1% pyruvate [23]. Liquid cultures were incubated at 32°C with shaking at 200 rpm, and solid culture plates were incubated at 32°C.

DNA Manipulations and Sequence Analysis

DNA was manipulated using standard protocols [26]. Oligonucleotides were synthesized at Bioneer Co., and DNA sequencing was carried out at Takara Korea Biomedical Inc. Preliminary sequence data of the *agiA* locus of DK1622

Table 1. Strains and plasmids used in this study.

Strains and plasmids	Relevant features	Source or references
M. xanthus		
DZ2	Wild-type	[6]
DZ4469	DZ2, Δ*pilA*, A^+ S^-	[33]
DK1217	*aglB1*, A^- S^+	[9]
KYC101	DZ2::pKY301-101, *agiA101*	This study
KYC464	DZ2::pCY103, *agiA103*	This study
KYC465	DZ2::pCY104, orf3	This study
KYC472	DZ2::pCY121, orf2	This study
KYC473	DZ2, Δ*agiA126* created using pCY126 in DZ2	This study
KYC475	DZ2, Δ*agiA126* Δ*pilA* created using pCY126 in DZ4469	This study
KYC499	*aglB1* Δ*pilA*, A^- S^-	This study
Plasmids		
pZErO-2	Cloning vector, Km^R	Invitrogen
pCY103	pKY468 carrying a 408-bp internal DNA fragment of *agiA*	This study
pCY104	pKY468 carrying a 412-bp internal DNA fragment of orf3	This study
pCY121	pKY468 carrying a 494-bp internal DNA fragment of orf2	This study
pCY126	Derivative of pKY480 used to create the Δ*agiA126* mutation	This study
pKY301	A library of plasmids containing about 500-bp DNA fragments from DZ2	[7]
pKY301-101	pZErO-2 carrying a 292-bp internal DNA fragment of *agiA*	This study
pKY468	Cloning vector, Km^R	[7]
pKY480	Cloning vector, $SacB^+$, Km^R	[7]

was obtained from The Institute for Genomic Research through the Web site at http://www.tigr.org. Basic Local Alignment Search Tool (BLAST) was used for homology searches [2]. Artemis was used to identify open reading frames (ORFs), DNASIS Max (MiraiBio) was used to analyze protein sequences, and Simple Modular Architecture Research Tool (SMART) was used to analyze functional domains of proteins [27].

Plasmid Construction

KYC101 carries a kanamycin-resistant plasmid, pKY301-101, inserted in the *agiA* gene. pKY301-101 was obtained by transforming *E. coli* DH5α to a kanamycin-resistant strain with the genomic DNA of KYC101. pCY103 is a derivative of pKY468, carrying a 408-bp internal DNA fragment of *agiA*. The internal DNA fragment was PCR amplified using two oligonucleotides, 5'-GCACTCGAG-CGGCTATGGCTGGTACTCGG-3' and 5'-GCAGGATC-CTACAGCAACGAGCTGGAGCG-3', as primers and the genomic DNA of DZ2 as a template, respectively. The PCR product was inserted into the XhoI and BamHI sites of pKY468, creating pCY103. pCY104 is a derivative of pKY468, carrying a 412-bp internal DNA fragment of *orf3*. The internal DNA fragment was PCR amplified using two oligonucleotides, 5'-GCACTCGAGGCTGGCAAGT-CCACCACCTT-3' and 5'-GCAGGATCCGGTGTGCCC-AGAAGGTACGG-3', as primers and the genomic DNA of DZ2 as a template, respectively. The PCR product was inserted into the XhoI and BamHI sites of pKY468, creating pCY104. pCY121 is a derivative of pKY468, carrying a 494-bp internal DNA fragment of orf2. The internal DNA fragment was PCR amplified using two oligonucleotides, 5'-GCACTCGAGAGGAACATGATGGCGAAGAG-3' and 5'-GCAGGATCCGACCTGATCTCCCTCACCAA-3', as primers and the genomic DNA of DZ2 as a template, respectively. The PCR product was inserted into the XhoI and BamHI sites of pKY468, creating pCY121. pCY126, which was used to create an in-frame deletion of *agiA* (*ΔagiA126*), was constructed by the reported method [12, 17, 18]. Four oligonucleotides were used to amplify a PCR fragment carrying an *agiA* in-frame deletion: 5'-GACCTCGAGGGATAACAAAGCCCGGATG-3' (N1), 5'-ACAGGATCGTACTGCGACCGTGAAGCAAAGGCG-TGTTCAAG-3' (N2), 5'-ACGGTCGCAGTACGATCCT-

GTCTCGAAGGACTCGAAGCAGG-3' (C1), and 5'-GACGGATCCGCGTTCACACAGACTCCAGA-3' (C2). Primers N1 and N2 were used to amplify a 465-bp N-terminal PCR fragment, and primers C1 and C2 were used to amplify a 467-bp C-terminal PCR fragment. The resultant two PCR products and primers N1 and C2 were then used to amplify a final 911-bp PCR product, carrying an *agiA* in-frame deletion. The final PCR fragment was then ligated into pKY480 after digestion with BamHI and XhoI to generate pCY126.

Creation and Characterization of Mutants

Plasmid insertion mutations were performed with the method described previously [7, 29]. Fruiting body development of *M. xanthus* was initiated by spotting 20 μl of 5×10^9 cells/ml on a CF plate containing 1.5% agar. Spreading of swarms of cells on the agar surface was measured 24 h after a 2-μl spot of 5×10^8 cells/ml was placed on CYE plates containing 0.3% or 1.5% agar. Fruiting body development and spreading of swarms of cells were observed with a Nikon SMZ1000 stereomicroscope, and myxobacterial cells were observed with a Nikon E600 phase-contrast microscope. Still images were captured with a Nikon Coolpix-995 digital camera. Gliding movement of cells was captured with a Samsung Techwin SHC-721 CCD camera and recorded using a Samsung SVR-960D time-lapse video recorder.

RESULTS AND DISCUSSION

Identification of a Locus Required for Gliding Motility of *M. xanthus*

As an effort to identify the genes required for the gliding motility of *M. xanthus*, we carried out random plasmid insertion mutagenesis with the plasmid library pKY301. The pKY301 library has more than 5,000 random DNA fragments with the length of approximately 500 bp, which were originated from the sheared chromosomal DNA of the wild-type strain *M. xanthus* DZ2 [7]. Since pKY301 cannot replicate autonomously in *M. xanthus*, transformants for kanamycin resistance were selected after introducing the library into *M. xanthus* DZ2, thereby resulting in growth of cells in which the plasmid DNA was randomly integrated into the chromosome by homologous recombination. Among the 5,000 kanamycin-resistant colonies screened, 11 showed phenotypes indicating altered motility on 1.5% agar plates. The sites of plasmid integration were then mapped by determining the sequence of the DNA fragment in the inserted plasmid, which were recovered as described in Materials and Methods. BLAST search of the resultant DNA sequences in the nucleotide database at the National Center for Biotechnology Information (NCBI) revealed that a mutant, KYC101, had a plasmid insertion in a locus that was previously unidentified, whereas other mutants had insertions in loci that were already identified by other researchers, such as *aglT*, *agmK*, *agmP*, *agmU*, *agmW*, *difE*, *pilA*, *pilN*, *pilR*, and *tgl*. *aglT*, *agmK*, *agmP*, *agmU*, and *agmW* are known to be involved in A-motility, whereas *difE*, *pilA*, *pilN*, *pilR*, and *tgl* are known to be involved in S-motility [30, 37].

Sequence and Mutational Analysis of the *agiA* Locus

DNA sequence analysis indicated that KYC101 had a plasmid insertion in an ORF (Fig. 1) located 7.3 kb upstream of the *cspB* gene, which was previously reported [36]. To verify that the plasmid insertion in this ORF was responsible for the phenotype of KYC101, we created another plasmid insertion mutant, KYC464, by introducing pCY103 carrying a 408-bp internal DNA fragment of this ORF into the wild-type strain, DZ2 (Fig. 1). We also created an in-frame deletion mutant, KYC473, in which 92.5% of the DNA of this ORF was deleted (Fig. 1). When KYC464 and KYC473 were placed on vegetative (CYE) and starvation (CF) media, they showed a mutant phenotype that was similar to that of the KYC101, confirming that the insertion in this ORF was responsible for the mutant phenotype of KYC101. Since this ORF is required for the adventurous gliding motility of isolated cells, as described below, this ORF was designated *agiA* (adventurous gliding motility of isolated cells). There were four ORFs (orf2–orf5) downstream of *agiA* (Fig. 1), which were predicted to encode proteins for a putative ATP-binding cassette (ABC) transporter. Since these ORFs were transcribed with the same direction as the *agiA*, it is possible that these ORFs constituted an operon with *agiA* and are functionally related to *agiA*. Therefore, we tested whether the null mutation of orf2 and orf3 would give a phenotype similar to the *agiA* null mutation. However, when KYC465 and KYC472 carrying plasmid insertions in orf3 and orf2, respectively, were placed on CF and CYE media, they showed the wild-type phenotype. Consequently, it was concluded that the disruption of the *agiA* gene is solely responsible for the mutant phenotype of the KYC101 strain.

Sequence analysis predicted that *agiA* encodes a putative 787-amino-acid-long protein with eight tripeptide repeat (TPR) motifs (Fig. 2). The amino acid sequence of a region (400–773) was 21% identical to *O*-linked *N*-acetylglucosamine transferase (OGT) from humans, which also has multiple

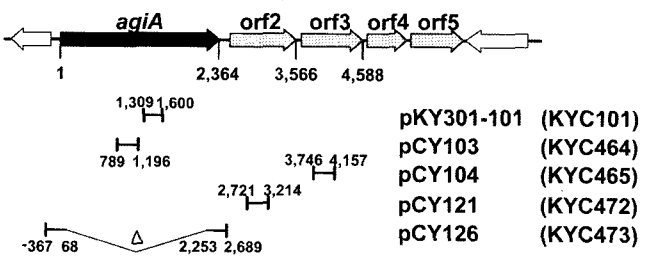

Fig. 1. Physical map of the *agiA* locus and the plasmids used for mutant generation.

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                                                                I
MSISSSKTLSPAELAKLEHAFASDPSSAAYKPLAEAYLSMGRFMEAMVVC  50
                                                  II
KKGVKAHPNAADPRLLLARVYAEQGKDKKALEEALGALQVQPEDKVALRM 100

AGALQLKTGEAEPGKANLLKAYTADPGDPDTVTLLQQHKIEPPRPAAPAP 150

VAAPAAPVAPATPAPSATQQSASALSSVAATAGTEAPAAKPAATQAAKPA 200

PTARAEAPAPRPAPQPRRPQVVVEEVDEDEDDEPVSRRSPSSGGKGSKMV 250

TLGLLVAIPLFAIGYGWYSANARTKSRELKKSLDVASELLKHDSFDSYKK 300

AVEAADQALEVDSDSAVAHGYLAYAWAIRWGEHGGGDDARRQAEEHLAVG 350

KKSGDLSSHLIASEALIQTYSGKGKDALGQLEEKVKGLDAQGRSSSLLYL 400
                  III                              IV
TLGLIQMNAGDLERGRDSLDRAQLLSPDDPRTYASLGAVYRRLGQDAAAW 450

RNYDSALRYGKDHPESLLGRSLLMLEQDSPNFELAHTMLKKLLESDPPPS 500

PRQLAAAHMARSLLISRVSTAMVDLKPDMQQKLAEATAVPQDKDKARTEM 550
                                    V
LKSEETGFSLDKQNPELHLIKGRRLLAEGNFDAAAEEIRKAIRMDASRAQ 600

FYVELAKSLMGKQGGEKDAAEALQTALKTMGDSPKLVVMLGNAYRRQGKL 650
VI                                  VII
DEALAQYQRAVKDPKAKNPEARLAMGAIYRERSKWDLAQEQLEKASQEFL 700
                        VIII
GQSDRAAIALTELGRVFQSKGDTAKADETYQRALNADEAYSPAYYFYATL 750

LSKDSKQGAKAKMLAQEYLKREPSGEHASAARSLAGG               787
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Fig. 2. Amino acid sequence of the putative AgiA protein and its domain organization. Boxed sequences are TPR domains predicted by the SMART program and the Roman numbers indicate the numbers of the predicted TPR domains.

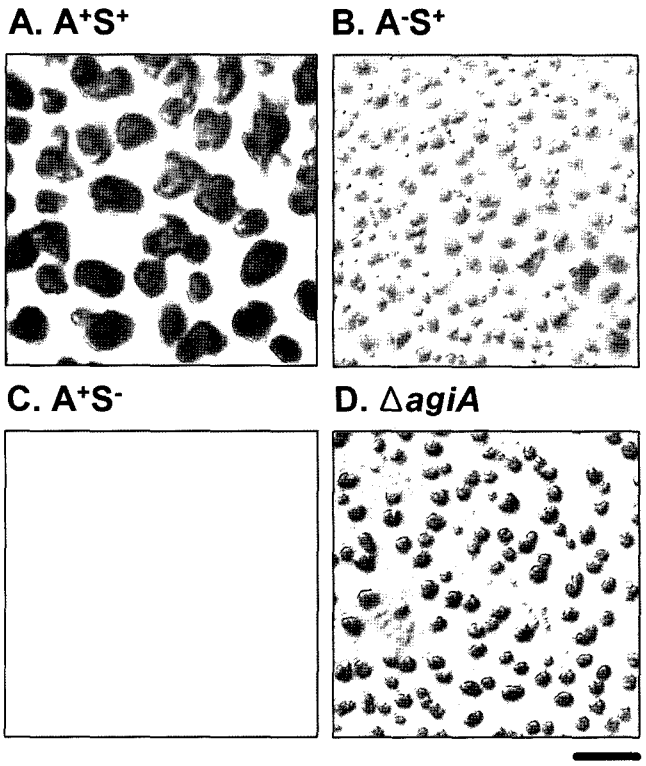

Fig. 3. Effect of the *agiA* null mutation on fruiting body development.
Twenty-μl spots (5×10^9 cells/ml) of DZ2 (**A**, wild-type), DK1217 (**B**, A^-S^+), DZ4469 (**C**, A^+S^-), and KYC473 (**D**, Δ*agiA*) were placed on CF plates and incubated at 32°C for 3 days. Bar, 1 mm.

TPR motifs [15]. The same region also showed similarity to the putative OGT from other organisms, although similarities were low (21–24% identical). In addition, the same region was 24% identical to AglT, which is one of the A-motility proteins [37]. The *agiA* appeared to have its own promoter, since the ORFs upstream of *agiA* were transcribed divergently from *agiA* (Fig. 1).

Effect of the *agiA* Null Mutation on Fruiting Body Development

The *agiA* null mutant, KYC473, was defective in fruiting body development (Fig. 3D): It formed mounds of cells that were much smaller than those of the wild-type strain, DZ2, and the sporulation efficiency of the mutant was less than 20% of the wild-type. Since the mutant was defective in motility, as described below, it implies that the *agiA* mutant is defective in fruiting body development because of the defect in motility, which is essential for the fruiting body development of *M. xanthus*.

Effect of the *agiA* Null Mutation on Gliding Motility

M. xanthus has two gliding motility systems: A-motility and S-motility. Shi and Zusman [27] found that *M. xanthus* cells that are defective in A-motility but retain normal S-motility (A^-S^+) spread more slowly on the surfaces of hard agar (1.5%) plates than on soft agar (0.3%) plates, whereas those that are defective in S-motility but intact in A-motility (A^+S^-) show the opposite phenotype [28]. When the *agiA* in-frame deletion mutant, KYC473, was placed on 1.5% and 0.3% agar plates, the swarm of mutant cells spread very slowly on 1.5% agar plates, as shown in Fig. 4H, whereas the swarm spread efficiently on 0.3% agar plates, as shown in Fig. 4D. This, therefore, implied that the *agiA* mutant might be defective in A-motility.

One of the representative characteristics of the A-motility defective mutant is the formation of smooth-edged colonies without a periphery of motile single cells, as shown in Fig. 5B. On the other hand, as shown in Fig. 5C, S-motility mutant cells disperse at the edges of colonies. The edge of colonies of the *agiA* mutant, KYC473, appeared to be smooth, and no single cell apart from the swarm was observed (Fig. 5D), similar to that of the A-motility mutant. This result also suggested that the *agiA* mutant might be defective in A-motility.

To confirm whether the *agiA* mutant was defective in A-motility, we examined the gliding movement of isolated cells on agar surface: A-motility mutants are known not to glide individually apart from other cells. Thus, if the mutant is defective in A-motility, the isolated *agiA* mutant cells

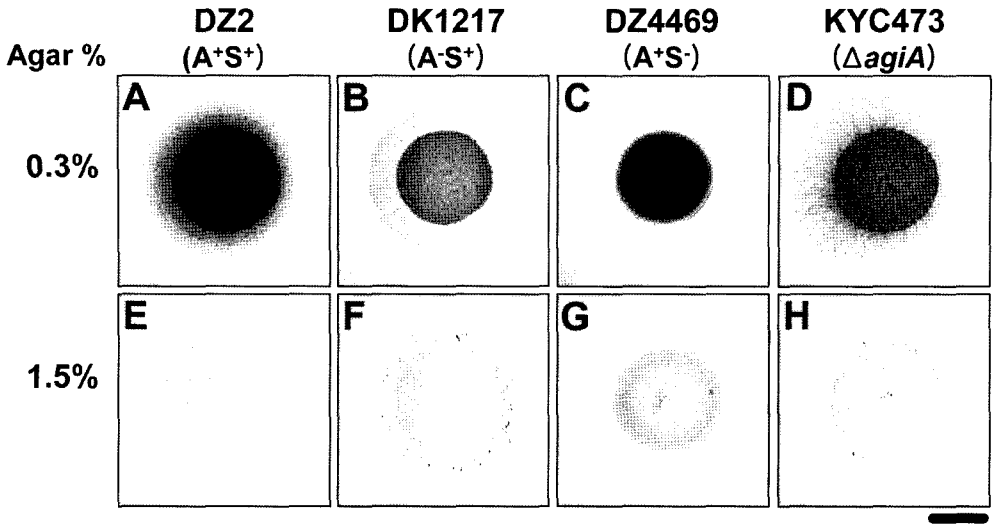

Fig. 4. Effects of the *agiA* mutation on the spreading of swarms of cells.
Two-μl spots of cell suspension (5×10^8 cells/ml) were placed on CYE plates containing 0.3% agar (**A–D**) and 1.5% agar (**E–H**) and incubated at 32°C for 48 h. Panels: **A** and **E**, DZ2 (A^+S^+); **B** and **F**, DK1217 (A^-S^+); **C** and **G**, DZ4469 (A^+S^-); and **D** and **H**, KYC473 (Δ*agiA*). Bar, 2 mm.

would not move. Since the gliding speed of *M. xanthus* was too slow to observe directly, we recorded the movement of cells under a microscope with a time-lapse video recorder and analyzed it. When the diluted wild-type cells (DZ2) were spread on a slide glass covered with CF agar, the isolated cells moved normally. On the other hand, the isolated A-motility mutant cells (DK1217) did not move at all. Then, under the same condition, the isolated *agiA* mutant cells (KYC473) also did not move at all, confirming that the *agiA* mutant is defective in A-motility.

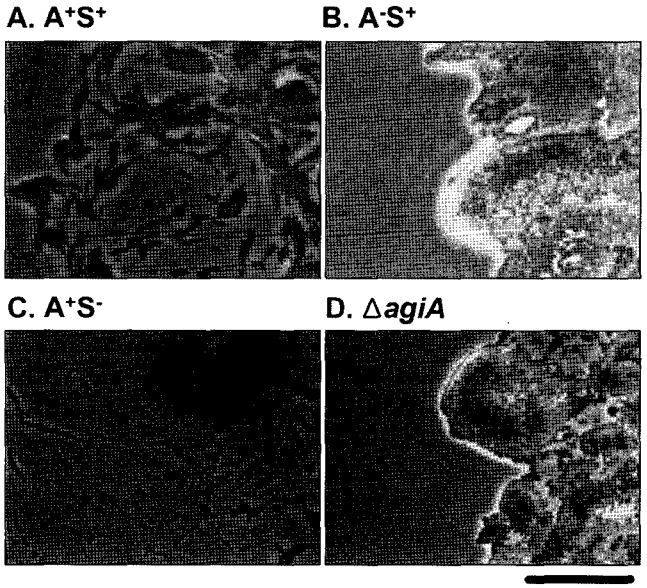

Fig. 5. Effect of the *agiA* mutation on the behavior of individual cells at the edge of colonies.
Two-μl spots of cell suspension (5×10^8 cells/ml) were placed on CYE plates containing 2% agar and incubated at 32°C. Pictures of the edge of colonies were taken after 24 h. Panels: **A**, DZ2 (A^+S^+); **B**, DK1217 (A^-S^+); **C**, DZ4469 (A^+S^-); and **D**, KYC473 (Δ*agiA*). Bar, 50 μm.

The *agiA* mutant, KYC473, was unable to glide individually when the cells were isolated from other cells, but they were gliding normally in a swarm of cells, indicating that S-motility of the mutant was intact (*agiA*$^-$ S$^+$). This suggests that the *agiA* mutation causes defects in A-motility, but not in S-motility.

A- and S-motilities are genetically separable. Thus, the mutant defective only in A-motility retains S-motility (A^-S^+), and the mutant defective only in S-motility displays A-motility (A^+S^-). On the other hand, the mutant defective in both A- and S-motilities becomes nonmotile (A^-S^-). To confirm further whether the mutant was really defective in A-motility or not, it was necessary to show that the *agiA* mutant created in the S$^-$ mutant background was nonmotile. Thus, we created KYC475 (*agiA*$^-$ S$^-$) by introducing the *agiA* mutation into the S-motility mutant, DZ4469 (A^+S^-), and also created KYC499 (A^-S^-) by introducing an A$^-$ mutation, *aglB1*, into the same S-motility mutant, DZ4469, as a control strain. On the agar surface, the S-motility mutant cells (DZ4469) moved normally, leaving trails of slime behind them (Fig. 6A) since they have the A-motility system. However, the KYC499 (A^-S^-) neither moved nor produced slime trails (Fig. 6C). Under the same condition, KYC475 (*agiA*$^-$ S$^-$) did not move at all. We tried the same experiment under several different conditions; nevertheless, we could not observe any gliding movement of the mutant cells. In addition, no slime trail formation was observed (Fig. 6B). KYC475 also failed to form fruiting bodies. Based on these results, we conclude that the *agiA* mutant is defective in A-motility, and that the *agiA* gene is required for the adventurous movement of isolated cells.

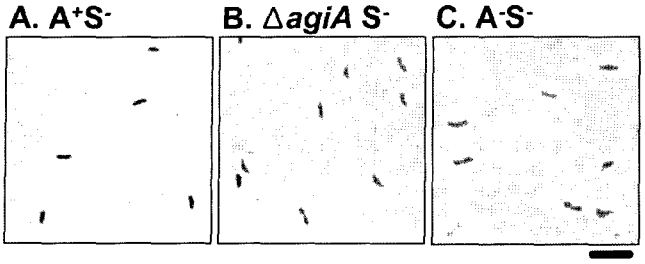

Fig. 6. Effect of the *agiA* mutation on the gliding movement and simile trail formation of isolated cells.
Five-μl cell suspensions (5×10^8 cells/ml) were spread on glass slides covered with CF agar media and incubated at room temperature. Movement of individual cells and formation of slime trails were recorded at different time points. Panels: **A**, DZ4469 (A^+S^-, after 30 min); **B**, KYC475 (Δ*agiA* S^-, after 5 h); and **C**, KYC499 (A^-S^-, after 5 h). Bar, 10 μm.

The A-motility system functions whenever cells are in a group or not, whereas the S-motility system functions only when cells are in group. Therefore, as shown in Fig. 7, DZ4469, which is defective only in S-motility (A^+S^-), moved in a swarm of cells, resulting in continuous variation of the shape of the swarm. Under the same condition, the shape of the swarm of KYC499, which is defective in both A- and S-motilities (A^-S^-), did not change, since the cells did not move at all in a swarm (Fig. 7). However, it was surprising to observe that the shape of the swarm of KYC475 (*agiA*$^-S^-$) varied continuously, as shown in Fig. 7: The *agiA* mutant cells were gliding in the swarm, indicating that, although the isolated KYC475 cells (*agiA*$^-S^-$) cannot move individually, they have an ability to glide in the swarm of cells.

In this study, we identified the *agiA* gene that appears to be another A-motility gene. The isolated mutant cells carrying null mutations in *agiA* were defective in adventurous gliding movement. More than 34 A-motility genes have so far been identified, and it is quite possible that there are more A-motility genes not yet identified [25, 31, 34, 37]. However, *agiA* is different from other known A-motility genes in that the *agiA* mutant created in the S^- mutant background glides in the swarm of cells. Since the *agiA* mutant displays a unique motility phenotype, further studies on this mutant would provide valuable information on the gliding mechanism of *M. xanthus*.

Acknowledgments

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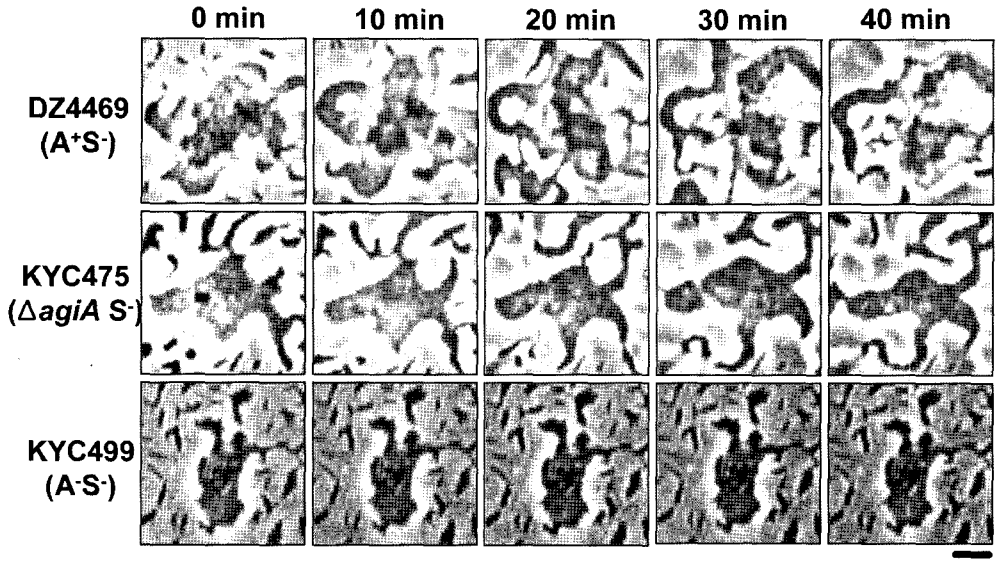

Fig. 7. Gliding movement of the *agiA* mutant in swarm of cells.
Fifty-μl cell suspensions (5×10^8 cells/ml) of DZ4469 (A^+S^-), KYC475 (Δ*agiA* S^-) and KYC499 (A^-S^-) were placed on CF agar media. Movement of the swarm of cells was recorded at room temperature. Bar, 10 μm.

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