Involvement of two novel proteins in *Mycoplasma mobile* gliding machinery revealed by newly developed gene manipulation and fluorescent protein tagging

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ABSTRACT

*Mycoplasma mobile* has a unique mechanism that enables it to glide on solid surfaces faster than any other gliding mycoplasma. To elucidate the gliding mechanism efficiently, we developed a transformation system for *M. mobile* based on a transposon derived from Tn4001. Modification of electroporation conditions, outgrowth time, and colony formation from the standard method for *Mycoplasma* species enabled the successful transformation. Then, a fluorescent-protein tagging technique was developed based on enhanced yellow fluorescent protein (EYFP) and was applied to two proteins whose involvement in the gliding mechanism has been suggested. P42 (MMOB1050), transcribed as continuous mRNA with other proteins essential for gliding, and a homolog of F1-ATPase α-subunit (MMOB1660) were shown to localize at the position of the gliding machinery. Analysis of the amino acid sequence of P42 by PSI-BLAST suggested similarity with FtsZ, the bacterial "tubulin" and that P42 developed from a common ancestor with FtsZ and achieved a special role. The roles of these proteins are discussed as part of our hypothesis for the gliding mechanism.

GENERAL INTRODUCTION

*Mycoplasma*

Members of the class *Mollicutes*, which include *Mycoplasma, Spiroplasma,* and *Achromoplasma,* are parasitic or commensal bacteria featuring reduced genome sizes (560 to 2300 kb). Phylogenetically, *Mollicutes* belong to the high-AT branch of gram-positive bacteria, which also includes *Clostridium* and *Bacillus* (1). Unlike the cells of other bacterial groups, *Mollicute* cells lack a peptidoglycan layer and are covered with membrane-anchored proteins, including surface proteins responsible for antigenic variation (2). The firstly isolated mycoplasma in culture was *Mycoplasma mycoides* subsp. *mycoides*, the bovine pleuropneumonia agent, was described initially in 1898. Since then, about 200 species of mycoplasmas have been discovered from vertebrate including human, arthropods, and plants. These species are classified phylogenetically into four subgroups, *hominis, pneumoniae, spiroplasma,* and *phytoplasma,* on the basis of their rRNA and the genome sequences.

A large number of mycoplasma species are effective pathogens of humans and wide range of animals in which they often cause chronic infections that result in morbid rather than mortal disease. These simple bacteria have adopted a parasitic lifestyle and live in close contact to
the host, with a predilection for the mucosal surface of the respiratory and genital tracts. While current advances in molecular biology, genomics and proteomics continue to take advantage of their reduced genome to understand broad biological concepts, factors involved in mycoplasma-host colonization, virulence and pathogenesis remain largely unknown (3).

**Gliding motility of mycoplasma**

Gliding motility is a striking feature which is thought to be involved in pathogenicity of certain *Mycoplasma* species. Gliding mycoplasma species form a membrane protrusion at a cell pole and exhibit gliding motility in the direction of the protrusion (4). They can glide on various solid surfaces such as glass, plastic, and animal cells. Several mycoplasma species including *Mycoplasma pneumoniae*, *M. gallicepicum*, *M. genitalium* and *M. mobile* exhibit gliding motility. These species belong to two phylogenetic groups, i.e., the *hominis* subgroup represented by *M. mobile* and the *pneumoniae* subgroup, both of which are distantly positioned phylogenetically (2).

The gliding motility of mycoplasmas is different from the cell motility systems of eukaryotic or prokaryotic cells known so far. *Mycoplasmas* do not have flagella, pili, or any homologs of known mechanisms of bacterial motility. In addition, there are no reported homologs of conventional motor proteins, which are usually involved in eukaryotic motility (4). The surface motility of bacteria, excluding *Mycoplasmas*, is classified into two major types; (i) Flagellar swimming motility generates motion by the rotation of flagellar filaments and is highly suitable for aqueous environments. However, in viscous environments or on solid surfaces with limited water content, flagella-based motility may not function efficiently. To move in these environments, bacteria such as the spirochetes have sequestered their flagella within the periplasmic space; when the flagella rotate, the spiral-shaped cell bodies rotate and can corkscrew their way through viscous fluids. In contrast, many bacterial species, when placed in a viscous medium, amplify the number of flagella on the cell surface; these hyperflagellated swarmer cells can then swarm efficiently over surfaces (Fig. 1a). However, some bacterial species have evolved alternative motility mechanisms that allow cells to move on solid surfaces without the aid of flagella (5). (ii) Some bacteria such as *Mycobacterium xanthus*, *Flavobacterium johnsoniae*, *M. mobile* exhibit gliding motility. In gliding motility, cells crawl over surfaces, but the proteins known to be involved in cell movement in each are unrelated to conventional motor proteins. *M. xanthus* has two motility systems, referred to as Social (S) and Adventurous (A) motility systems. S-motility involves type IV pilus extension
and retraction, as does bacterial twitching motility (6). Twitching motility is a flagella-independent form of bacterial translocation over moist surfaces. Twitching motility is important in host colonization by a wide range of plant and animal pathogens. A-motility appears to be driven by motors in the cell envelope that harvest the proton gradient and exert force on proteins in the periplasm. Cargo proteins propelled within the periplasm along a helical track, causing localized deformation of the peptidoglycan layer and outer membrane, resulting in cell movement (Fig. 1d). A difference between *M. xanthus* and *F. johnsoniae* motility is that in the former, motor proteins have been proposed to move long distances within the cell, whereas in the latter they are thought to be anchored within the cell envelope to be able to propel cell-surface adhesins (Fig. 1f) (7).

![Fig 1. Types of bacterial motility. a) Swimming motility powered by the rotation of flagellar filaments. b) Sheathed flagella-driven motility by spirochetes suitable in highly viscous fluids. c) Swarming motility on a solid surface powered by multiple lateral flagellar filaments. d) Social gliding motility resulting from the retraction of type IV pili adhered to a solid surface or other bacterial cell bodies. e) Gliding motility of mycoplasma. f) Gliding motility of *Flavobacterium johnsoniae.*](image)

**Gliding of Mycoplasma mobile**

*Mycoplasma mobile* is one of the flask-shaped mycoplasmas (∼1 μm × 0.3 μm) and was originally isolated from a fish, the tench (*Tinca tinca*) (8). It belongs to the *Mycoplasma hominis* group of the mycoplasma phylogeny, which also includes two other sequenced species (*Mycoplasma pulmonis*, and *Mycoplasma hyopneumoniae*). It is believed to be pathogenic and grows optimally at around 25°C, somewhat lower than most other well-studied mycoplasmas, whose optima are around 37°C, yet its doubling time (∼10 h) is within the range of mycoplasmas with mammalian hosts. However, *M. mobile*’s single most defining characteristic is its ability to glide on solid surfaces. *M. mobile*, is the fastest
mycoplasma species with an average speed of 2.0 to 4.5 μm per second, or 3 to 7 times the length of the cell per second, exerting a force up to 27 pN (Fig. 2) (9).

Transformation of mycoplasmas

Transformation of mycoplasmas was not clearly demonstrated until 1987 when Dybvig and Cassell were able to show the introduction of the Gram-positive transposon Tn916 into Acholeplasma laidlawii and Mycoplasma pulmonis. This was the first evidence that mycoplasma could be transformed. These studies have led to numerous attempts to develop mutagenesis schemes, as well as cloning vectors for gene analysis and other functions in mycoplasmas (10).

Mycoplasmas are structurally simple prokaryotes lacking cell walls. With only a lipid membrane bilayer to penetrate, one would presume that they would be easily transformed or genetically manipulated. For instance, mycoplasmas are incapable of taking up naked DNA. Moreover, the systems that are available for mycoplasma transformation are laborious, time-consuming, and inefficient ((11),13). And also, because of the low transformation frequencies, typical transformation schemes used with mycoplasmas require large amount of DNA. Therefore, transformation technique of individual Mycoplasma species is different from each other. The three common methods to transform bacteria, chemical treatment with CaCl₂ or polyethylene glycol (PEG), liposome-mediated delivery of encapsulated DNA, and electroporation-based procedures have all been tried in mycoplasmas (12). Transformation of different species of mycoplasmas with these techniques showed that electroporation is the most flexible and highest efficiency method.

Fig 2. Optical and electron microscopy images and gliding trace of M. mobile cells, about 1 μm long.
Electroporation

Electroporation is the application of controlled direct current (DC) electrical pulses which are applied to living cells and tissues for a short duration of time. The pulse induces a transmembrane potential which causes the reversible breakdown of the cellular membrane. This action results in the permeation or “pore formation” of the cell membrane which allows small molecules (such as dye, oligonucleotides or peptides) and large molecules (such as proteins, DNA and RNA) to be introduced into the cell (13). During this process the cellular uptake of the molecules continue until the pores close which can take milliseconds to minutes. There are two types of pores created during electroporation, transient and long-lived, and both play a role in transport. The permeable state can last up to minutes after the application of the electric pulse, and by overcoming this barrier a variety of molecules can diffuse or be electrophoretically driven through the destabilized membrane. The permeabilization of the membrane is transient and the membrane reseals itself after the voltage is removed. If electric fields are too high, the cell membrane is disrupted beyond repair and results in cell death (14).

Negatively charged DNA is electrophoretically driven through the destabilized membrane while the pulse is applied. If long-lived pores remain open after the pulse is removed DNA can continue to diffuse through the permeable membrane. Higher electric fields lead to larger permeabilized area of cell membrane, due to higher energy for pore formation, resulting in more pores being formed. Transient pores also increase with increasing number of pulses, suggesting each pulse contributes to more/larger stable long-lived transport pores (13, 14).

Pulse shape generally falls into two categories, square wave or exponential decay wave:

Square wave pulse: Square wave pulses rise quickly to a set voltage level, maintains this level during the duration of the set pulse length and quickly turns off. This square wave system yields higher efficiencies and viabilities in mammalian cells. Square wave EP in vivo and ex vivo tissues, embryo’s, cell fusions and plant protoplast applications yield better results in comparison to an exponential decay wave system.

Exponential decay wave pulse: Exponential decay waves generate an electrical pulse by allowing a capacitor to completely discharge. A pulse is discharged into a sample the voltage rises rapidly to the peak voltage set then declines over time. The powerful exponential decay wave pulse is routinely used for transformation of gram-negative and gram-positive, bacterial, yeast, plant tissues, insect cells and some mammalian cells (Fig. 3) (15).
There are some issues that should be considered when performing electroporation:

**a) Field Strength:** The field strength is measured as the voltage delivered across an electrode gap and is expressed as kV/cm. Field strength is critical to surpassing the electrical potential of the cell membrane to allow the temporary reversible permeation or “pore formation” to occur in the cell membrane. Three factors should be considered for optimizing field strength:

i. **Cuvette Gap Size.** The distance between electrodes, or “gap size” is important when optimizing the electroporation experiment. Field strength is calculated using voltage divided by gap size. It is possible to derive the voltage needed to accomplish electroporation if the desired field strength and gap size are known.

ii. **Cell Diameter.** Generally, smaller cell sizes require higher voltages while larger cell diameters require lower voltages for successful cell membrane permeation.

iii. **Temperature.** The temperature at which cells are maintained during electroporation effects the efficiency of the electroporation for several reasons. Samples which are pulsed at high voltage or exposed to multiple pulses and long pulse durations can cause the sample to heat up. These conditions cause increased cell death and lowers the transformation efficiency. Maintaining the sample at lower temperatures can diminish the heating effects on cell viability and efficiency. Since electroporation causes the transient formation of pores, keeping the cells at lower temperature following the pulse may allow the pores to remain open longer to allow more uptake of the exogenous molecule.

**b) Pulse Length:** The pulse length is the duration of time the sample is exposed to the pulse. This is measured as time in micro to milliseconds ranges. The pulse length in an exponential decay wave system is called the “**time constant**” which is characterized by the rate at which the pulsed energy (e) or voltage is decayed to 1/3 the original set voltage. This time constant is modified by adjusting the resistance and capacitance (RC) values in an exponential decay. Time constant calculation $\tau=RC$, where $\tau$ is time and $R$ is resistance.

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**Fig 3.** Exponential decay wave and square wave pulse types
and C is capacitance. The pulse length works indirectly with the field strength to increase pore formation and therefore the uptake of target molecules. Generally, during optimization of parameters an increase in voltage should be followed by an incremental decrease in pulse length. Pulse length is a key variable that works hand in hand along with voltage and needs to be considered when optimizing electrical parameters to maximize the results for a given cell type (13).
INTRODUCTION

Mycoplasmas are commensal and occasionally parasitic bacteria that lack peptidoglycan layers and have small genomes (2). Mycoplasma mobile, a fish pathogen, forms a membrane protrusion at one pole and exhibits gliding motility in the direction of the protrusion (4, 8, 16). The average speed is 2.0 to 4.5 μm/s, or three to seven times the length of the cell per second, exerting a force of up to 27 pN (17-19). This motility, combined with the ability to adhere to the host cell surface, likely plays a role in parasitism, as has been suggested for another species, Mycoplasma pneumoniae (4, 16, 20-22). This motility has been shown not to be related to other mechanisms of bacterial movement, nor does it involve motor proteins known to be involved in eukaryotic cell motility (9).

We have been studying the gliding mechanism of M. mobile since 1997 and clarified the following facts. The cell surface can be divided into three parts from the front end, i.e. head, neck and body as shown in Fig. 4A (4, 23, 24). Three huge proteins, Gli123, Gli349, and Gli521, with respective masses of 123, 349, and 521 kDa are involved in this gliding mechanism and are localized at the cell neck (23-27). Electron microscopy showed that 50 nm legs are sticking out from the neck surface, which is composed of Gli349 (Fig. 4B) (28-30). The surface structure should be supported from cell inside by a unique cytoskeleton designated "jellyfish structure" whose ten components have been identified by mass spectrometry (Fig. 4C) (31). The energy for motility is supplied by ATP (32, 33), and the direct binding target is sialylated oligosaccharide found on animal tissue surfaces in nature (34, 35).

On the basis of above information, we proposed a working model, called "centipede" or "power-stroke" model, where cells are propelled by “legs” composed of Gli349 that, through repeated cycles, catch and release sialylated oligosaccharides based on ATP energy (4, 36). However, to obtain a complete image of this gliding mechanism, much more information is necessary.
**Fig 4.** Proteins focused on in the present study are boxed. (A) Cell inside structure marked "i" (upper) and surface structure marked "s" (lower). Cytoskeletal "jellyfish structure" can be divided into two parts, "bell" and "tentacle". Bell and tentacles are composed of protein products of MMOB1670, and MMOBs 1630 and 4860, respectively. The cell surface can be divided into three parts, head, neck, and body from the front end. Gliding direction is indicated by a black arrow. Neck is covered by Gli123, 349, and 521 proteins, essential for gliding. Head and body are covered with Mvps C, E, F, N, O, I and Mvps C, K, I, which are likely involved in evading mechanism from host immune system. (B) Magnified image of neck surface, based on our previous studies mainly by electron microscopy. The distal globular part of Gli349 is thought to catch and pull sialylated oligosaccharides fixed on host surface, which is driven by ATP energy. (C) ORFs for surface (upper) and jellyfish (lower) structures of gliding machinery. Ten ORFs have been identified as the components of jellyfish structure and four ORFs are coded at other loci on the genome.
Although 10 proteins have been identified as the component of jellyfish structure, their roles and subcellular localization are still unclear (31). Interestingly, the amino acid sequences of components, MMOB1660 and MMOB1670 show high similarity to the $\alpha$- and $\beta$-subunits, respectively, of F1 ATPase, the catalytic subunit of proton pumps (31, 37). However, these proteins are unlikely to function in a proton pump, because the proton pump of *Bacillus subtilis* requires an additional seven subunits (38), and the *M. mobile* genome has another locus containing the complete set of pump subunits. P42 protein is coded tandemly with Gli123, Gli349, and Gli521 essential for gliding and has been suggested to be transcribed together with these three Gli proteins as a continuous mRNA (26), but its role and subcellular localization are unknown (39).

In microbiological studies in general, gene manipulation is a powerful tool with which to clarify the function and roles of genes and proteins. However, this strategy has been hampered due to the complete lack of a genetic system for manipulating the *M. mobile* genes.

In the present study, I identified the causes inhibiting the transformation of *M. mobile*, solved all of them, and achieved transformation. I also achieved fluorescent protein tagging, and elucidated the subcellular localization of two key proteins plausibly involved in the gliding mechanism.
MATERIALS AND METHODS

Strains and culture conditions. *M. mobile* 163K (ATCC 43663) and *M. pulmonis* (ATCC 19612) strains were cultured in Aluotto medium at 25°C and 37°C, respectively (40, 41). For the selection of transformed *M. mobile*, tetracycline hydrochloride, gentamicin sulfate, chloramphenicol, or puromycin dihydrochloride was used with a final concentration of 3.0, 30, 18, or 2.5 μg/ml, respectively. *Escherichia coli* strain DH5α was used for DNA manipulation.

Plasmid construction. *M. mobile* genomic DNA was prepared by the Genomic-tip System (Qiagen, Hilden, Germany). pTK165 was kindly provided by Tsuyoshi Kenri at the National Institute of Infectious Diseases, Tokyo, Japan (42). Plasmid pMobtuf was constructed by replacing the *M. pneumoniae* tuf promoter sequence (upstream from the *eyfp* gene) by the tuf promoter amplified from the *M. mobile* genome using BamHI and NcoI sites in the plasmid. The *eyfp* gene (derived from plasmid pEYFP, Clontech, Palo Alto, CA) of pMobtuf was replaced with a codon-optimized sequence (accession number: AB860250) from pMD19-Myco plasmid, which was kindly provided by Itaru Yanagihara at Osaka Medical Center for Maternal and Child Health, Osaka, Japan, using the NcoI and EcoRI sites in pMobtuf plasmid, producing pMobopt plasmid. Plasmids containing p42 (MMOB1050) and MMOB1660 fusion genes were constructed as follows. For N-terminal fusion of p42, the DNA fragment amplified from the genomic DNA by PCR was inserted into the 5’ end of the *eyfp* gene of pMobopt by using the In-Fusion EcoDry PCR Cloning Kit (Takara Bio, Shiga, Japan), producing pMobN-P42 plasmid. For C-terminal fusions, p42 (MMOB1050) and MMOB1660 genes amplified from the genomic DNA were inserted into the 3’ end of the *eyfp* gene of plasmid pMobopt by the In-Fusion EcoDry PCR Cloning Kit, producing plasmids pMobC-P42 and pMobC-1660, respectively.

Transformation of *M. mobile*. The transformation procedure was modified from the method generally used for mycoplasma (43). The major modification points were harder conditions for electroporation, longer outgrowth time to grow the cells in medium without antibiotics after electroporation, and lower concentration of serum included in the solid medium. *M. mobile* was grown in liquid medium until the mid-logarithmic phase (~10^8 CFU/ml). Cells were collected by centrifugation at 9000 × g at 4 °C for 10 min and washed three times in an equivalent volume of electroporation buffer (272 mM sucrose, 8 mM HEPES, pH 7.4). The cells were then suspended in 100 μl of electroporation buffer at a concentration of approximately 10^9 cells per ml, kept on ice with 10 μg of plasmid DNA for 30 min, and then transferred to a pre-chilled 2 mm electroporation cuvette. Electroporation
was performed using Gene Pulser II and Pulse Controller II (Bio-Rad Laboratories, Hercules, CA). Optimized electroporation conditions--2.5 kV, 800 Ω, and 25 μF--were applied. Immediately after electroporation, 900 μl ice-chilled Aluotto medium was added. After incubation for 15 min on ice, the cells were kept at 25°C for 12 h for outgrowth. Aliquots of 100 μl were plated onto solid Aluotto medium containing 6% horse serum and appropriate antibiotics, and were cultured at 25°C for 8-9 days. The plates were observed by an SZX2-ILLT stereo microscope (Olympus, Tokyo, Japan) and photographed. To isolate the transformants, individual colonies in agar were picked and homogenized by a BioMasher (Iwai Chemicals, Tokyo, Japan).

**Colony PCR.** Each colony was picked and homogenized, and used as the template. PCR was performed with primers, 5'- ATGAATATAGTTGAAAATGAAATATGTATAAG-3' and 5'- TCCTTTAATTTTATAACCTAGTATAGAT -3', complementary to the gentamicin-resistant gene. The PCR conditions were: (i) 5 min at 95°C, (ii) 30 cycles of shifts, 1 min at 95°C, 30 s at 55°C, 1 min at 68°C, (iii) 68°C for 5 min. PCR products were checked using the Multina microchip electrophoresis system (Shimadzu, Kyoto, Japan).

**Microscopy.** To observe EYFP fluorescence, individual M. mobile transformants were picked and grown in liquid Aluotto medium containing 15 μg/ml gentamicin at 25°C for 3 days. The cells were collected by centrifugation at 12,000 × g for 4 min, suspended in fresh medium, and bound to coverslips washed with saturated ethanolic KOH. The cells were then fixed by 3.0% paraformaldehyde and 0.1% glutaraldehyde for 30 min at RT and observed with a BX51 fluorescence microscope equipped by a YFP filter unit (U-MYFPHQ) and a phase-contrast setup (Olympus, Tokyo, Japan) (23, 42, 44). Immunostaining and DAPI staining fluorescence microscopy and the observation of gliding were performed as described previously (17, 23-25, 44).

**Transposon insertion mapping.** The DNA sequences flanking transposon insertion sites were determined using arbitrary PCR (45, 46). The genomic DNA of transformant was isolated by the agarose block method, as previously described (47). A DNA fragment covering an end region of a transposon and the flanking region of insertion sites was amplified by a nested PCR. The first-round PCR was performed to isolate genomes using a primer unique to the right end of the Tn4001 element, 5'- CTTTTACACAATTACCGGACTTTATC -3' and an arbitrary primer, 5'-GGCCACCGCGTCGACTAGTACNNNNNACGCC -3'. The PCR conditions were: (i) 10 min at 95°C; (ii) 6 cycles of shifts 30 s at 95°C, 30 s at 30°C, 1.5 min at 72°C with 5 s increments per cycle; and (iii) 30 cycles of shifts 30 s at 95°C, 30 s at 45°C, 2 min at 72°C with 5 s increments per cycle. The second-round PCR was performed to obtain
the first-round product using primers, Tn4001-IR-R-in (5'-GGACTGTATATGGCCTTTTTAACTTTTACACA -3') and 5'-GGCCACGGCTCGACTAGTAC-3. The second-round PCR conditions were: 30 cycles of shifts, 30 s at 95°C, 30 s at 45°C, 2 min at 72°C. All PCR products were recovered from the agarose gel, and sequencing was carried out using BigDye v3.1 with Tn4001-IR-R-in primer by a 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). The sequence reads obtained were aligned against the M. mobile whole genome sequence (9).

Existence of original p42 gene and MMOB1660 on the genome was confirmed by PCR of regions covering the whole gene and one flanking region. The primer sequences used were 5'-GATGTACCTTCAGGAGCAATTATTG-3', 5'-TTATCTTAGAAGAATTTCTTCTGTCAAG-3', 5'-ATGACAAAAAATTGAAATTTCGAAAAT -3', and 5'-GATAACTATCATTTTTITTATCTTAGAAG-3' for p42, and 5'-GTAATTGTACCTATTGCAATTATTTG-3', 5'-TTATTGTAGTCATATGCTATACCTCTTTCC -3', 5'-ATGAAAAATTTAAAAATAACAGCAATTAAAG -3', and 5'-CTAATTCTGTAAGATTATTTGTAGTCATAT -3' for MMOB1660.

Measurement of ATP. Cell suspensions of 50 μl before and after electroporation were centrifuged at 12,000 × g at 4 °C for 4 min. The pellets were separated from the supernatant, resuspended in 50 μl electroporation buffer, and applied to ATP Bioluminescence Assay kit HS II (Roche, Basel, Switzerland) (48, 49). The emission was measured by Varioskan Flash (Thermo Scientific, Waltham, MA).

Protein analysis. M. mobile cells were grown in tissue culture flasks to the mid-log phase. The cells were collected by centrifugation at 12,000 × g for 4 min at 4°C and washed three times with phosphate-buffered saline (PBS) consisting of 75 mM Na-phosphate (pH 7.3) and 68 mM NaCl. Cells were lysed by adding sample loading buffer and were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). For immunoblot analysis, the separated proteins were transferred to a nylon membrane (Bio-Rad Laboratories). The EYFP was detected by a monoclonal antibody specific for A. victoria GFP variants (Clontech Laboratories, Mountain View, CA) and a horseradish peroxidase (HRP)-conjugated secondary antibody (42).

Sequence analyses. Sequence similarity was searched by PSI-BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) against NCBI nonredundant protein sequences. The sequences of homologs were aligned by T-COFFEE
(http://www.igs.cnrs-mrs.fr/Tcoffee/tcoffee_cgi/index.cgi), and their phylogenetic tree was made by ClustalW (http://www.genome.jp/tools/clustalw/).

**Accession number.** The sequence of *eyfp* gene whose codon was optimized for expression in mycoplasma is available from INSD under accession number, AB860250.
RESULTS

Achievement of transformation. In my initial attempts, I tried unsuccessfully to transform *M. mobile* by transposon vectors used in other mycoplasma species (Table 1) (10, 42, 50, 51), through the electroporation under the conditions 2.5 kV, 100 Ω, 25 μF, and a cuvette with a 2 mm gap, which are generally used for mycoplasma transformation (43). I then examined and improved each step of the transformation procedure.

<table>
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<th>Plasmid</th>
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<th>Efficiency (transformants/cfu/μg DNA)</th>
<th>Days to colony formation</th>
<th>Source</th>
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<td>2.5E-04</td>
<td>9</td>
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First, I examined the permeabilization of the cell membrane after the electroporation by optical microscopy and on the basis of the ATP content remaining in the *M. mobile* cells. The results were compared with those of *M. pulmonis*, a relative of *M. mobile* that can be transformed (Fig. 5A, B) (9, 10, 54, 55). Optical microscopy showed that most of the *M. pulmonis* cells lost cell density, showing that the cell membrane was well permeabilized by the electroporation, but *M. mobile* cells did not show significant damage by microscopy. The ATP amount in cells was consistent with the result of microscopy. *M. pulmonis* cells lost 87% of their ATP content, but *M. mobile* lost only 13%. These results showed that *M. mobile* cells are more resistant to the applied electric shock than *M. pulmonis*. To permeabilize the *M. mobile* cell membrane to an extent similar to that in *M. pulmonis* cells, we varied the
resistance for electroporation from 100 to 800 Ω in 100 Ω increments. Applying 2.5 kV, 800 Ω, and 25 μF conditions resulted in a change in appearance and about an 80% loss of ATP on *M. mobile* cells, similar to the loss on *M. pulmonis* (Fig. 5A, B). Then, I modified the conditions of electroporation to transform *M. mobile*.

On solid growth medium, some mycoplasma species tend to form weblike structures called films and spots in prolonged cultivation (Fig. 5C). These structures are thought to be formed by the activity of lipases, which break down phospholipids derived from horse serum contained in the growth medium, and sometimes disturb colony formation (56, 57). In the formation of colonies without antibiotics, film and spot formation does not matter because *M. mobile* cells form colonies in 3 days. However, prolonged incubation is required to form colonies of transformants in the presence of antibiotics, owing to their slow growth rate, even if they are resistant to the antibiotics. I then tried lower concentrations of serum to reduce the film and spot formation, keeping the growth rate of *M. mobile* cells normal, and modified the serum concentration in the solid medium for transformation from 10% to 6%.

Next, I modified the outgrowth time, which is when the cells are grown in liquid medium without antibiotics to repair the damage caused by the electroporation and express the resistance gene. I tried different outgrowth durations, from 2 h to 16 h at 2 h increments, and finally succeeded in getting transformed colonies, after 7 to 9 days of incubation by modifying the outgrowth time to 8 h (Fig. 5D). The transformation was achieved by all transposon vectors tried. The efficiency was improved with the extension of outgrowth time to 12 h, and reached $10^{-4}$ to $10^{-5}$ transformants per CFU by 1 μg of DNA (Table 1), but efficiency did not improve with outgrowth times longer than 12 h. Then, I fixed the outgrowth time to be 12 h for transformation of *M. mobile*. 
Among the five vectors I tried here, I focused on pTK165 for further studies because it showed the highest transformation efficiency, $4.2 \times 10^{-4}$ transformants per CFU by 1 μg DNA, and gave the fastest growth rate, 7 days for colony formation (Table 1) on medium containing gentamicin. Transformation was confirmed by colony PCR of individual colonies randomly selected from solid media. These clones yielded the expected 805 bp PCR fragment using Tn4001-specific primers. The PCR detected no bands from the nontransformed M. mobile colonies. The transposon was stable for at least five consecutive inoculations on a solid medium with gentamicin.

Fluorescent protein tagging. To clarify the subcellular localization of interested proteins, I developed a fluorescent protein tagging method. As the pTK165 plasmid has been used for the same purpose in Mycoplasma pneumoniae, I examined the fluorescence of M. mobile transformants by fluorescence microscopy (42). However, the M. mobile transformants did not show fluorescence signals. I then replaced the promoter sequence of elongation factor Tu to express the eyfp gene, from the M. pneumoniae sequence to the M. mobile one, resulting in

Fig 5. Optimizing conditions for transformation. (A) Cell images of M. pulmonis and M. mobile after electroporation under different conditions. The cells were electroporated with the use of 100 Ω or 800 Ω resistances and were observed by phase-contrast microscopy. (B) ATP amounts in cells after electroporation under different conditions. The ATP amounts in cells without electroporation were normalized to 100%. (C) "Film and spot" formation under different concentrations of serum. The serum concentration applied to the solid medium is indicated in each panel. Film and spots cover the medium surface only in the left panel. The surface of the solid medium was observed 7 days after inoculation. (D) Colony formation at 7th day after inoculation.
a plasmid, pMobtuf (Fig. 6A) (9). The transformants then showed clear emission of fluorescence (Fig. 6B). To improve the fluorescence intensity, I tried the promoters of an abundant *M. mobile* surface protein, MvspI (48, 58, 59), and an abundant *Spiroplasma citri* surface protein, Spiralin (60). However, neither constructs showed any fluorescence signal. As mycoplasma genomes are biased toward high AT contents, the codon usage of mycoplasma genes is different from that of many other organisms. To improve the fluorescence intensity, I replaced the *eyfp* gene with one optimized for codon usage of mycoplasmas, resulting in the pMobopt plasmid, and achieved a 1.9-fold increase in fluorescence intensity (Fig. 6B,C).

Next, I chose the P42 protein as the initial target for the fluorescent protein tagging. P42 is coded in the same operon with three large proteins essential for gliding, suggesting its participation in the gliding mechanism (Fig. 6) (26, 39). However, even its subcellular localization is unknown. Then, the *p42* gene was fused to the 3' or 5' end of the *eyfp* gene, resulting in *p42-eyfp* and *eyfp-p42* fusion genes, respectively. I detected similar fluorescence intensity from pMobC-P42 with pMobopt, but I did not detect a signal from pMobN-P42. In all cells transformed by pMobC-P42, the signals were found at the neck part (Fig. 6B and 8).

The next target protein was MMOB1660, which is a F1 ATPase ω-subunit homolog and a component of jellyfish structure (Fig. 1). In the present study, the MMOB1660 gene was fused to the 5' end of the *eyfp* gene, resulting in MMOB1660-*eyfp* fusion genes. The signal was found around the neck with a fluorescence intensity slightly higher than that of pMobC-P42 (Fig. 6B and 8).
**Fig 6. EYFP and its fusion protein expression examined by fluorescence and immunoblotting.**

(A) Design of constructs. The constructs listed here were inserted into an arm of a transposon in a transposon vector, pTK165 (31). Plasmid names are on the left. P: promoter of *M. pneumoniae* *tuf* (P_{tuf}) and that of *M. mobile* (P_{Mtuf}). NcoI, BamHI, and EcoRI recognition sites are shown as N, B, and E, respectively. Codon-optimized *eyfp* gene is marked *opt eyfp*. (B) Fluorescence images of cells. Phase-contrast and fluorescence images are merged. The cells were transformed by the plasmid indicated in the corresponding line in (A). (C) Quantification of fluorescence intensity. Average of more than 10 cells is presented with SD. (D) Immunoblotting analysis of EYFP and fusion proteins. Whole cell lysates were analyzed by SDS-10% PAGE (left) and blotting (right) for cells transformed by none (lane1).
I next examined the expression levels of EYFP and fusion proteins by immunoblotting (Fig. 6D). EYFP was not detected in the transformants harboring pTK165, but was detected in all constructs containing the *M. mobile tuf* promoter. The protein amounts were significantly influenced also by the protein fusion. The amounts of EYFP and fusion proteins were roughly consistent with the fluorescence intensity.

**Mapping of insertion sites on the genome.** Insertion sites were decided for 18, 10, and 10 isolates transformed by pTK165, pMobC-P42, and pMobC-1660, respectively (Fig. 7). Typically, 200-300 bp of DNA sequence flanking the insertion site was obtained by the nested arbitrary PCR followed by sequencing. The insertion sites were all different and distributed mostly around the genome, although four ORFs were identified as the insertion sites more than once. The analyses of 6 isolates for each by PCR suggested that the original genes of *p42* and MMOB1660 remains in the original position on the genome. The binding and gliding activities of transformed cells did not differ obviously from those of the wild type strain, suggesting that the fused proteins may function similarly with the original proteins, in these cases.

![Fig 7](image)

**Fig 7.** Transposon insertion sites on the genome. ORFs coded on top and bottom strands are presented by solid and gray arrows, respectively. The integration sites are shown by symbols specific for three plasmids with an ORF code. Positions on the genome are shown by nucleotide numbers in the circle.
Subcellular localization of P42 and F1 ATPase α-subunit homolog. To determine the subcellular localization of P42, the positional relationship between P42 and other structures were examined (Fig. 8). Gli349, MvspI, DNA localized at neck, head and body, and body, in good agreements with previous studies (Fig. 3A) (23, 24, 58, 61). P42 colocalized with Gli349, suggesting that P42 is also involved in the gliding machinery and mechanism. Next, I compared the subcellular localization of MMOB1660, the homolog of F1 ATPase α-subunit with that of Gli349, and concluded that MMOB1660 is also localized at the same position with that of Gli349, suggesting that MMOB1660 is a component of jellyfish tentacle.

Fig 8. Subcellular localization of P42 and the F1-ATPase α subunit homolog, MMOB1660. Localization of P42, MMOB1660, Gli349, DNA and MvspI were examined by fluorescence microscopy. P42 (upper three lines) and MMOB1660 (bottom line) were detected as EYFP fusion proteins. Gli349 and MvspI, and DNA were detected by immunostaining and DAPI staining, respectively. Phase contrast and fluorescence images were merged in the two panels at right. A single cell in the left panel in merged images is magnified in the right panel.
**Sequence analysis of P42.** The previous analyses of the amino acid sequence of P42 showed no clear similarity to any proteins other than the plausible ortholog (MYPU2170) of *M. pulmonis*, a close relative of *M. mobile* (9, 39). In the present study, I searched for similarities in by PSI-BLAST using the amino acid sequences of P42 and MYPU2170. This analysis gave a list of 39 similar proteins with e-values ranging from 0.025 to 1.5, of which 36 were assigned as FtsZ of gram positive bacteria, mainly *Clostridium* and *Bacillus*, related to *Mycoplasma*. Generally, 24 amino acids mostly involved in GTPase activity are well conserved in FtsZ and tubulin (62). Interestingly, however, only four of those amino acids are conserved in P42, suggesting that this protein does not perform the dynamic polymerization and depolymerization observed in FtsZ and tubulin (54). The phylogenetic tree analyzed with 12 related proteins showed that P42 and MYPU2170 form a group apart from FtsZs and most related to FtsZ of *M. pulmonis*. These results may suggest that P42 originated from the same ancestor with FtsZ and developed to have different functions.
DISCUSSION

Transformation of *M. mobile*. I found that *M. mobile* cells are more resistant to electric shocks than the other *Mycoplasma* species, consistent with our daily observations that the cell shape of *M. mobile* is more stable than the shapes of other species under a change in osmotic pressure. This feature of *M. mobile* cells may be caused by the regular array of gliding proteins on the surface of the gliding machinery (4, 26).

Transformants grow more slowly than nontransformants in growth medium without antibiotics, i.e., the colony formation takes 7 days with gentamicin, although it takes 3 days without antibiotics, and the growth rate did not depend on the insertion site of the transposon on the genome. These facts suggest that the slower growth rate of transformants is not caused by the disruption of important genes; instead, it might be caused by the growth reduction by antibiotics, even if they are transformed by the marker gene. In other words, the marker genes are not effective enough to recover the normal growth rate. These features can be seen also in the transformants of other *Mycoplasma* species. In the case of *M. pneumoniae*, it takes 4 days to form the colonies of transformant under antibiotics, but only 2 days without antibiotics (43). This may be caused by the insufficient expression of marker gene product or insufficient protein activity in a mycoplasma cell. The outgrowth of *M. mobile* took 12 hours, much longer than the 2-3 hours required for other *Mycoplasma* species. This difference may be related to the lower growth temperature of *M. mobile*, 25°C, which is distinct from other species. The expression of the gentamicin gene in an *M. mobile* cell may need more time because of the lower growth temperature.

Fluorescence intensity of EYFP. Plasmid pTK165, which has been used for fluorescent protein tagging in *M. pneumoniae*, did not provide sufficient fluorescence intensity in *M. mobile* cells (42). I then replaced the promoter of *M. pneumoniae tuf* by that of *M. mobile*, and also optimized the codon of the eyfp sequence to that of *M. mobile*. These two modifications provided sufficient fluorescence intensity, probably as a result of the higher levels of protein expression as shown by the immunoblotting analysis. The fluorescence intensity and the amount of fusion proteins depended on the construct, suggesting that the stability of a fusion protein is another determinant of fluorescent protein tagging. The fluorescence intensity did not totally parallel to the protein amount, showing that the environments around the fluorescence moiety are also critical.

Whole cell image. To date, subcellular localization has been clarified for 13 proteins (Fig. 3) (4, 31, 58). In the present study, two other proteins have been shown to localize on the
gliding machinery (Fig. 8). P42, has been suggested to have developed from an ancestor common with FtsZ to have a special role in the gliding mechanism. This protein should be localized cell inside or at an occluded position on the surface, because a previous study of biotinizing surface proteins did not detect exposure of P42 (58).

MMOB1660, an F1 ATPase α-subunit homolog and a component of the jellyfish structure, also has been shown to localize at a position similar to that of Gli349. As the position of Gli349 corresponds to that of the jellyfish tentacles (17, 31), we concluded that MMOB1660 localizes at the tentacles of the jellyfish structure. Previously, the MMOB1670 protein, an F1 ATPase β-subunit homolog, was shown to localize at the the jellyfish tentacles by immunofluorescence microscopy (31). Probably, these two proteins form a complex similar to F1 ATPase on the jellyfish tentacles. On the tentacles of the jellyfish structure, particles about 10 nm wide are aligned periodically with a pitch of about 30 nm. These particles may be composed of MMOBs 1660 and 1670.

**Gliding mechanism.** The experimental data showed that the gliding occurs by the repeated catch-pull-release of sialylated oligosaccharide by Gli349 coupled with the movements of Gli521, driven by ATP hydrolysis (Fig. 3) (4, 8, 36). However, the generation and transmission of movements are still unknown. In the present study, we suggested that a homolog of the F1 ATPase α-subunit forms the tentacles of the jellyfish structure with a homolog of F1 ATPase β-subunit. This may suggest that the movement for gliding is generated here by ATP hydrolysis. P42 may not conserve the dynamics of FtsZ because of its lack of amino acids essential for GTPase, but the surfaces needed for protofilament formation may be conserved. This protein may play a role in supporting or bridging parts in the gliding machinery, and may also be involved in transmitting the movements of the cell inside to the surface structures, including the "legs".

![Fig 9. Schematic model of *M. mobile* gliding energy source.](image)
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**Supp. Table 1.** Transposon insertion sites on the genome.