Role of the *Mycobacterium marinum* ESX-1 Secretion System in Sliding Motility and Biofilm Formation

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*Mycobacterium marinum* is a close relative of *Mycobacterium tuberculosis* that can cause systemic tuberculosis-like infections in ectotherms and skin infections in humans. Sliding motility correlates with biofilm formation and virulence in most bacteria. In this study, we used a sliding motility assay to screen 2,304 transposon mutants of *M. marinum* NTUH-M6885 and identified five transposon mutants with decreased sliding motility. Transposons that interrupted the type VII secretion system (T7SS) ESX-1-related genes, espE (*mmar_5439*), espF (*mmar_5440*), and eccA1 (*mmar_5443*), were present in 3 mutants. We performed reverse-transcription polymerase chain reaction to verify genes from *mmar_5438* to *mmar_5450*, which were found to belong to a single transcriptional unit. Deletion mutants of espE, espF, espG (*mmar_5441*), and espH (*mmar_5442*) displayed significant attenuation regarding sliding motility and biofilm formation. *M. marinum* NTUH-M6885 possesses a functional ESX-1 secretion system. However, deletion of espG or espH resulted in slightly decreased secretion of EsxB (which is also known as CFP-10). Thus, the *M. marinum* ESX-1 secretion system mediates sliding motility and is crucial for biofilm formation. These data provide new insight into *M. marinum* biofilm formation.

Keywords: type VII secretion system, *Mycobacterium marinum*, ESX-1, sliding motility, biofilm formation

INTRODUCTION

*Mycobacterium marinum* is a non-tuberculous photochromogenic mycobacterium. It is a close relative of *Mycobacterium tuberculosis*, and also causes systemic tuberculosis-like infections in ectotherms (Solomon et al., 2003; Hagedorn and Soldati, 2007; Alibaud et al., 2011). Researchers first isolated *M. marinum* from saltwater fish in the 1920s and identified it as a human pathogen in the 1950s (Riera et al., 2015). This species usually occurs in warm saltwater, freshwater, and poikilothermic animals; fish, frogs, and amphibians are its main natural hosts. It grows best at a temperature of 25–35°C (McArdle et al., 2016). The prevalence of *M. marinum* infections in humans has risen in recent years because of the increasing popularity of home aquariums (Riera et al., 2015). *M. marinum* infections most commonly occur on the skin, especially the extremities, because of the low-temperature requirements of the bacteria for growth (Oh et al., 2015). An *M. marinum* skin infection is referred to as an aquarium granuloma, swimming pool granuloma, or fish tank granuloma (Solomon et al., 2003; Meijer, 2015; Sette et al., 2015).

*M. marinum* infection usually occurs following contact with an infected animal or handling of contaminated aquariums or water. It can also occur as an opportunistic infection, primarily in...
Clinicians characterize the stages of *M. marinum* infections as the initial stage (type I), in which there are single or multiple skin papules or nodules; advanced stage (type II), in which there are granulomas; and severe stage (type III), in which immunosuppressed patients experience tenosynovitis, arthritis, or osteomyelitis (Riera et al., 2015). The diagnosis of *M. marinum* infection is often delayed, due to its low prevalence or because there are few specific clinical signs and symptoms. Thus, many patients initially receive incorrect diagnoses and inappropriate treatments.

Many mycobacteria can spread on a surface by sliding (also called “growth-powered passive surface translocation”), which is driven by the outward pressure of cell growth. This process, which does not require flagella (Martinez et al., 1999), occurs due to surfactants, which decrease the surface tension and allow the spread of cells from their origin (Kearns, 2010). Several studies have indicated that lipooligosaccharides (LOSs), glycolipid dimycocerosates (PDIMs), and phenolic glycolipids (PGs) on the outer surface of mycobacteria have important functions in sliding motility (Ren et al., 2007; Tatham et al., 2012; Pang et al., 2013; Mohandas et al., 2016).

Sliding motility allows the colony diameter of non-swarming bacteria to increase during prolonged incubation (Kearns, 2010). Mycobacteria are prototypical non-flagellated microorganisms that spread slowly in a uniform monolayer due to sliding motility (Shi et al., 2011; Maya-Hoyos et al., 2015). Furthermore, sliding movements and biofilm formation facilitate diffusion and colonization by mycobacteria (Pang et al., 2013).

A biofilm is a thin and slimy film of one or more species of bacteria that adhere to each other and/or a solid surface and it can help to increase the virulence of the bacterial species. Biofilm formation may be considered a survival “strategy” for bacteria. There are several consecutive stages of biofilm formation: reversible attachment, irreversible attachment, mature biofilm formation, and dispersion. A biofilm consists of bacteria and matrix material, which includes extracellular polymeric substances, such as polysaccharides, lipids, membrane vesicles, and nucleic acids (Sousa et al., 2015; Toyofuku et al., 2015). Bacteria in biofilms have significantly enhanced resistance to antibiotics and the human immune system (Shi et al., 2011). Biofilm formation is highly related to sliding motility in *Mycobacterium* spp. (Nesar et al., 2011; Mohandas et al., 2016). Previous studies indicated that *Mycobacterium smegmatis* with defects in biofilm formation also have impaired sliding motility (Recht and Kolter, 2001). In addition, Ghosh et al. (2013) reported that the ability of *M. smegmatis* to form biofilms declined as sliding motility declined. Many other species of mycobacteria, including *Mycobacterium avium* and *Mycobacterium fortuitum*, are well-known to produce biofilms, although the ability of *M. marinum* to form biofilms remains largely unknown (Ren et al., 2007).

There has been an increased number of cases of *M. marinum* infection due to the increasing popularity of home aquariums. However, there is little knowledge about the pathogenic mechanism of this species in humans. Moreover, sliding motility is highly associated with biofilm formation in mycobacteria. In this study, we aimed to identify genes that have roles in sliding motility in *M. marinum*.

**MATERIALS AND METHODS**

**Bacteria Strains**

*M. marinum* NTUH-M6885 is a strain that was clinically isolated at the National Taiwan University Hospital. It was cultured in 7H9 medium supplemented with 10% oleic acid/albumin/dextrose/catalase (OADC), 0.5% glycerol, and 0.05% Tween-80 at 32°C (Tan et al., 2006; Chen et al., 2015). *Escherichia coli* DH10B was grown in Luria broth (LB). When required for the experiments, the antibiotic hygromycin was used at a concentration of 50 mg/L for *M. marinum* and 100 mg/L for *E. coli*.

**Generation of *M. marinum* Transposon Mutant Library**

*M. smegmatis* mc^2^155 was used to propagate the TM4-derived conditionally replicating phage phAE94 [which was a kind gift from Dr. William R. Jacobs, Jr., Howard Hughes Medical Institute, New York, NY, United States (Bardarov et al., 1997)]. This phage carries the kanamycin-resistance transposon Tn5367 (Shin et al., 2006). We followed the procedures used in previous studies (Rybniker et al., 2003; Chen et al., 2015) to promote phAE94 infection of *M. marinum* NTUH-M6885 cells.

**Screening for *M. marinum* Transposon Mutants With Decreased Sliding Motility**

Sliding motility mutations were screened for using a sliding agar plate (7H9, 6% glycerol and 0.3% agarose) (Mohandas et al., 2016). An aliquot of 1 µL bacteria culture (in the stationary phase) was dropped onto these agar plates (24- or 6-wells) and cultured at 32°C for 9–11 days. To confirm the sliding defect in transposon and deletion mutants, 1 µL bacteria culture adjusted to an optical density at 600 nm (OD_{600}) of 1 was dropped onto 6-well plates and cultured at 32°C for 7 days. The diameters of the sliding areas were then measured.

**Identification of Transposon Mutants by Semi-Random Polymerase Chain Reaction (PCR)**

The insertion sites of Tn5367 in the transposon mutants were identified by semi-random PCR and DNA sequencing as previously described (Chun et al., 1997; Choi et al., 2001; Shin et al., 2006; Chen et al., 2015). Supplementary Table 1 shows the primers used in these experiments.

**Construction of Deletion Mutants**

Gene-deleted fragments were constructed using primers listed in Supplementary Table 1. The Hygr-lacZ-sacB cassette of the...
pGOAL19 plasmid (Addgene plasmid #20190, Cambridge, MA, United States) was digested with Scal and the gene-deleted fragments were then cloned into the Scal site of the digested plasmid. We then transformed the constructed plasmid into *M. marinum* NTUH-M6885 by electroporation (BTX®, ECM 630 Electroporation System, MA, United States) under the following conditions: 2500 mV, 1,000 Ω, and 25 μF (Larsen et al., 2007). Following the procedure of Parish and Stoker (2000) and Chen et al. (2015), *M. marinum* unmarked deletion mutants (with in-frame deletions) were obtained after two rounds of homologous recombination. After recovering from the electroporation, each transformed *M. marinum* mutant was cultured on 7H11 with 50 mg/L hygromycin for 2 weeks. The single colony was then subcultured on 7H11 with 100 mg/L X-gal and 50 mg/L hygromycin for 1 week to select the single cross-over transformants (blue colonies). The blue colonies were selected and cultured on 7H11 with 2% sucrose and 100 mg/L X-gal for 1 week to obtain the deletion mutants (white colonies).

**5' Rapid Amplification of cDNA Ends (RACE)**

The 5’-RACE procedure was performed using a SMARTer™ RACE cDNA Amplification Kit (CA, United States). This kit includes SMARTer II A oligonucleotides and SMARTScribe Reverse Transcriptase. This allowed isolation of the complete 5’ sequence of our target transcript from the total RNA.

**Construction of Complemented Strains**

After isolating the complete 5’ sequence of the target operon, we amplified and connected the promoter region and target genes using overlap- or inverse-PCR, with primers listed in Supplementary Table 1. The complementation fragments were then cloned into a blunted HindIII-site of pMN437 (which was a kind gift from Dr. Michael Niederweis, University of Alabama at Birmingham, Birmingham, AL, United States) (Steinhauer et al., 2010). Subsequently, we transformed the complementation plasmid into deletion mutants.

**Growth Curve**

Cultures were inoculated with fresh precultures to an OD$_{600\text{nm}}$ of 0.1. Bacterial growth was monitored spectrophotometrically, and colony counts were determined every day.

**Biofilm Formation**

*M. marinum* was cultured in 7H9 medium (without Tween-80) with OADC, and with shaking at 100 rpm (Pang et al., 2012; Mohandas et al., 2016). The cell concentration was adjusted to an OD$_{600\text{nm}}$ of 0.01 in round-bottom 96-well polystyrene cell culture plates (costar® 3897, New York, NY, United States) at 32°C, and the cells were then cultured for 3 weeks. After 3 weeks, the medium was removed. We stained the biofilm with 200 μL 1% crystal violet (CV) for 10 min (Trivedi et al., 2016). We then removed the CV and washed the biofilm twice with 1× phosphate-buffered saline (PBS). Subsequently, we extracted the CV from the biofilm using 99.5% EtOH and assessed the biofilm formation by measuring OD$_{990\text{nm}}$.

**Confocal Laser Scanning Microscopy**

*M. marinum* was cultured on cover slides with 7H9 medium (without Tween-80) with OADC, and with shaking at 100 rpm for 2 weeks. The bacteria were then washed with PBS before fixing in formalin. The biofilm was observed with a Leica TCS SP5 confocal laser scanning microscope (Leica, Wetzlar, Germany) and three-dimensional images were analyzed using Velocity software (version 6.0.1, MA, United States).

**Western Blotting**

Bacteria were cultured in 7H9 medium for 4 days (to mid-log phase). We then removed the culture medium and washed the bacteria with Sauton’s medium. The bacteria were then cultured for 4 days at 32°C (Gao et al., 2004). Culture filtrates collected by centrifugation and filtration through 0.22 μm-pore-size polyethersulfone filter were condensed using a concentrator (Amicon, NJ, United States) with a 3-kDa cut-off. Next, 10 μg proteins were loaded on 15% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) gel. The proteins were detected using antibodies against 10 kDa culture filtrate protein (CFP-10; which is also known as ExsB), Ag85B (Ag85B protein levels was used for normalization), and heat-shock protein 65 (Hsp-65; also called grOEL; Hsp-65 protein levels was used for normalization). The details regarding the antibodies were as follows: anti-CFP-10 antibody (Abcam, ab45074, 1:5000), anti-Ag85B antibody (Abcam, ab43019, 1:3000), and anti-Hsp-65 antibody (grOEL; Abcam, ab20519, 1:200).

**Two-Dimensional Thin-Layer Chromatography (2D-TLC)**

The extraction and 2D-TLC analysis of polar lipids was based on established procedures used in our previous study (Chen et al., 2015) and in a study by Burguiere et al. (2005). First, the polar lipids were extracted from *M. marinum* grown on 7H11 agar plates. Second, lipids were examined using TLC aluminum sheets (Merck, Summit, NJ, United States). Third, the LOS signals were visualized by spraying the plates with ceric ammonium molybdate [CAM; 24 g (NH$_4$)$_6$Mo$_7$O$_{24}$·4H$_2$O, 0.5 g ammonium cerium nitrate, 500 mL H$_2$O, and 28 mL H$_2$SO$_4$], followed by gentle charring of the plates (Chen et al., 2015).

**Statistical Analysis**

All the data were from three independent experiments and they are presented as means ± standard deviations (SDs). We estimated the statistical significance of the differences using one-way analysis of variance (ANOVA) or two-tailed Student’s *t*-tests using GraphPad Prism software (version 5.01, La Jolla, CA, United States).
RESULTS

Screening an M. marinum Transposon Library for Sliding Motility

We first constructed a transposon library of M. marinum NTUH-M6885 containing 2,304 mutants to screen for genes associated with sliding motility. We then randomly selected 16 of these mutants and used semi-random PCR and sequencing to characterize them. The results showed that these 16 mutants had unique transposon insertion sites (Supplementary Table 2), indicating that the library had good diversity.

Next, we screened the 2,304 mutants for sliding motility using 24-well sliding agar plates. We then re-examined and identified 13 mutants with defects in sliding motility using 6-well sliding agar plates. The wild-type strain slid to the edges of the culture well (3.5 cm in diameter), whereas the 13 mutants had sliding distances <2 cm (Supplementary Figure 1). Finally, we reperformed the same experiment using an equal quantity of bacteria cultured for 7 days (OD₆₀₀ = 1), and we found that only five mutants had sliding motility defects (Supplementary Figure 2).

Role of the Early Secreted Antigen 6 kDa (ESAT-6) Secretion System 1 (ESX-1) Genes in Sliding Motility

We identified the disrupted genes of the five transposon mutants with sliding motility defects using semi-random PCR and DNA sequencing (Table 1). Two mutants harbored insertions within tetR and phoU, which both belong to putative regulators. The other three transposon mutants—23-B1 (mmar_5439), 8-C7 (upstream of mmar_5440), and 22-B5 (mmar_5443)—had disruptions in the gene cluster encoding the type VII secretion system ESX-1 (Figures 1A,B). Previous studies named mmar_5439 as espE, mmar_5440 as espF, and mmar_5443 as eccA1 (Bitter et al., 2009; Sani et al., 2010). Among these gene candidates, we focused on the ESX-1 cluster containing espE, espF, and eccA1. The products of all three of these genes are supposed to be displayed at the cell surface but their functions regarding sliding motility have not been characterized in M. marinum. These initial results suggested that ESX-1-related genes could be involved in sliding motility.

| Mutant (library no.) | Genes inserted by transposon | Putative function |
|----------------------|-----------------------------|------------------|
| 12-B1                | mmar_4631                   | TetR family transcriptional regulator |
| 14-A7                | mmar_4859                   | Phosphate transport system regulatory protein PhoU |
| 23-B1                | mmar_5439                   | Secretion protein EspE |
| 8-C7                 | upstream of mmar_5440       | Secretion protein EspF |
| 22-B5                | mmar_5443                   | Type VII secretion AAA-ATPase EccA1 |

Detection of Growth Rates in Transposon Mutants With Sliding Defects

The presence of a surfactant in the culture medium and a high bacterial growth rate can increase sliding motility (Kearns, 2010). Thus, it is necessary to determine whether the impaired sliding of the transposon mutants resulted from a growth defect. Recovery of bacterial counts (data not shown) and monitoring changes in OD₆₀₀nm over time was carried out to measure the growth rates of the wild-type strain and its three transposon mutants (23-B1, 8-C7, and 22-B5). Based on assays in general culture medium (7H9 with 10% OADC, 0.5% glycerol, and 0.05% Tween-80) or sliding broth medium (7H9 with 0.5% glycerol), the growth rates of the three transposon mutants showed no significant differences compared to the corresponding growth of the wild-type strain (Figure 1C and Supplementary Figure 3A). Thus, the sliding defects of these three mutants were not due to defects in growth.

Transcriptional Units and Sequence Alignments of the ESX-1 Gene Cluster

Reverse-transcription (RT)-PCR to determine the transcriptional units of the ESX-1 gene cluster was performed with total RNA from M. marinum NTUH-M6885 as the template and primer pairs that hybridize within two consecutive genes. Total RNA without RT was used as the negative control, to exclude genomic DNA contamination. Figures 2A,B showed positive results for the junctions of genes from mmar_5438 to mmar_5450. In addition, Supplementary Figures 4A,B show the longer amplified PCR products containing two or three adjacent genes. The PCR products with expected sizes were also confirmed by sequencing. These results indicate that the ESX-1 gene cluster contains 13 genes that belong to a single transcriptional unit. This operon starts with mmar_5438 and ends with mmar_5450.

Next, we identified the 5’ start site of this operon using a SMARTer™ RACE cDNA Amplification Kit. The result showed that the transcriptional start site of the ESX-1 operon was located 120 bp upstream of mmar_5438 (Figure 2C). Furthermore, we determined the full DNA sequence of this operon in M. marinum NTUH-M6885 using next-generation sequencing. Analysis of the nucleotide sequences of the ESX-1 cluster from M. marinum NTUH-M6885 (National Center for Biotechnology Information [NCBI] accession number: MF034931) revealed 99% sequence similarity compared to the cluster from the M. marinum M strain.

Roles of espE, espF, espG, and espH Genes in Sliding Motility

The genes, espE (mmar_5439), espF (mmar_5440), and eccA1 (mmar_5443) are all located in the same operon. To further characterize whether the specific genes of the ESX-1 cluster play roles in sliding motility, unmarked deletion mutants of espE, espF, and eccA1 and also of espG (mmar_5441) and espH (mmar_5442), were constructed. The deletions of espE, espF, espG, espH, and eccA1 were validated by PCR using two primer pairs targeted to the deleted genes and their flanking regions (Supplementary Figure 5). Figures 3A,B show that the sliding motility of each unmarked deletion mutant, except for the eccA1
FIGURE 1 | Transposon disruption of three ESX-1-related genes significantly impairs sliding motility but not growth. (A) Transposon insertion sites within the ESX-1 locus. The three disrupted genes (gray) were espE (mmar_5439), espF (mmar_5440), and eccA1 (mmar_5443). The bold vertical lines indicate the transposon insertion sites. (B) Sliding motility of the three transposon mutants (8-C7, 22-B5, and 23-B1). Aliquots of 1 µL bacteria culture (OD$_{600}$ = 1) were added to 6-well sliding agar plates and cultured at 32°C for 1 week. (C) Bacteria were cultured in 7H9 medium supplemented with 10% oleic acid/albumin/dextrose/catalase (OADC), 0.5% glycerol, and 0.05% Tween-80. The growth of different strains was assessed based on OD$_{600nm}$. Here and below, all data are from three independent experiments and presented as means ± SDs with one-way ANOVA.

mutant (ΔeccA1), was dramatically reduced compared with the sliding motility of the wild-type strain. The motility defects of the gene deletion mutants were significantly restored by complementation with the corresponding gene (Figures 3C,D). The growth rates of these deletion mutants were not significantly different compared to the growth rate of the wild-type strain (Supplementary Figure 3B). However, the sliding motility of the espG-complemented strain was only partially restored by complementation with the plasmid containing the espG gene. As the ESX-1 operon contains the promoter for 13 genes, the results imply that proper regulation of espG gene expression might require cis-elements within the operon. Thus, these results suggest that espE, espF, espG, and espH have important roles in sliding motility in M. marinum NTUH-M6885.

Roles of espE, espF, espG, and espH Genes in Biofilm Formation

Previous studies indicated that sliding motility correlates with biofilm formation in Mycobacterium spp. (Nessar et al., 2011; Sousa et al., 2015; Mohandas et al., 2016). To discern whether the ESX-1-related genes contribute to biofilm formation, we compared the biofilm formation of the deletion mutants with that of the wild-type strain. After 3 weeks of culturing in general culture medium without Tween-80, the deletion mutants exhibited decreased biofilm formation compared with the wild-type strain. All complemented strains produced biofilms similar to that of the wild-type strain (Figure 4). To further confirm the role of these ESX-1 genes in biofilm formation, three-dimensional biofilms were formed on cover slides and they were analyzed using confocal laser scanning microscopy (Figure 5). The biofilms of these deletion mutants were thinner and more scattered than those of the wild-type strain, and complementation significantly restored biofilm formation. These data indicate that espE, espF, espG, and espH are required for biofilm formation in M. marinum.

EsxB Secretion in ΔespE, ΔespF, ΔespG, and ΔespH Mutants

According to previous studies, both EsxA (ESAT-6) and EsxB (CFP-10) are indispensable components of the ESX-1 secretion
FIGURE 2 | The sliding-related operon involves mmar_5438 to mmar_5450, and its 5' start site is 120 bp upstream of mmar_5438. The primer pairs were: 1, 5437-38 gap-F/5437-38 gap-R; 2, 5438-39 gap-F/5438-39 gap-R; 3, 5439-40 gap-F/5439-40 gap-R; 4, 5439-40 gap-F/5440-41 gap-R; 5, 5441-42 gap-F/5441-42 gap-R; 6, 5442-43 gap-F/5442-43 gap-R; 7, 5443-44 gap-F/5443-44 gap-R; 8, 5444-45 gap-F/5444-45 gap-R; 9, 5445-46 gap-F/5445-46 gap-R; 10, 5446-47 gap-F/5447-48 gap-R; 11, 5448-49 gap-F/5449-50 gap-R; 12, 5450-51 gap-F/5450-51 gap-R2. The primer list is shown in Supplementary Table 1. (A) Primer recognition sites. The bold horizontal lines indicate amplified gene fragments, and arrows indicate primers. (B) Data showing that the sliding-related operon involves mmar_5438 to mmar_5450. Each gel is independent and not cropped from different parts of the same gel. cDNA: cDNA of M. marinum NTUH-M6885 (template); DNA: DNA of M. marinum NTUH-M6885 (positive control); mRNA: mRNA of M. marinum NTUH-M6885 (negative control, to exclude genomic DNA contamination). (C) The 5' start site begins 120 bp upstream of mmar_5438 (gray). The bold vertical line indicates the 5' start site, detected using a SMARTer™ RACE cDNA Amplification Kit.
Four mutants (espE, espF, espG, and espH) have significantly impaired sliding motility. (A) Sliding motility of M. marinum deletion mutants (ΔespE, ΔespF, ΔespG, ΔespH, and ΔeccA1) and M. marinum NTUH-M6885 (wild-type) on sliding agar plates. (B) Quantification of sliding diameters of the 6 strains shown in (A). (C) Sliding motility of complemented strains relative to M. marinum NTUH-M6885 harboring pMN437 on sliding plates. (D) Quantification of sliding diameters of the nine strains in (C). The quantification of the sliding diameters was used for normalization, with the wild-type diameter being assigned a value of 1. Means and SDs from three independent experiments were calculated with one-way ANOVA (∗∗p < 0.01, ∗∗∗p < 0.001).

system (Ates et al., 2016; Wong, 2017) and they are considered to be indicators of the ESX-1 system with secretion function (Gao et al., 2004; Brodin et al., 2006; Champion et al., 2014). To investigate whether M. marinum NTUH-M6885 had a functional ESX-1 system, EsxB secretion from the wild-type strain was detected by western blotting. Cell filtrate of M. tuberculosis H37Rv was used as a positive control (Supplementary Figure 6). The M. marinum NTUH-M6885 wild-type strain and also all of the Esp-1 gene deletion mutants accumulated EsxB protein in their cell lysates. However, deletion of espE, espF, espG, and espH
led to 0.35-, 0.59-, 0.82-, and 0.73-fold lower secretion of EsxB protein than the secretion of the wild-type strain. These results indicate that wild-type M. marinum NTUH-M6885 could secrete EsxB protein. There was a substantial decrease in secretion for espG and espH deletion mutants, and a slight decrease for espE and espF deletion mutants.

**DISCUSSION**

The M. marinum NTUH-M6885 transposon mutant library was screened, and 5 mutants (8-C7, 12-B1, 14-A7, 22-B5, and 23-B1) that had defective sliding motility were identified. 12-B1 and 14-A7 had an interruption in tetR and phoU, respectively. Both genes are putative regulators and they might regulate the other downstream effector genes for sliding motility directly or indirectly. Further studies are needed to understand whether the regulation of tetR or phoU is critical for sliding motility in M. marinum. 22-B5 had a transposon insertion in the eccA1 gene and showed significantly decreased sliding motility; however, the sliding motility of the eccA1 gene deletion strain was similar to that of the wild-type strain. Several studies have reported that the insertion of transposable elements influences the expression of nearby genes (Wei and Cao, 2016) (which is known as the polar effect). The expression levels of genes both upstream and downstream of insertion sites can be affected.
FIGURE 5 | Visualization of biofilm formation on cover slides with confocal laser scanning microscopy (CLSM). To further analyze the structure of the biofilms, CLSM was performed using bacteria carrying pMN437, which harbors gfp. (A) Microscopic image at 20× magnification. Scale bar = 100 µm. (B) 3D imaging with a Z-stack of biofilm structure. 1 unit = 12.34 µm.
espE was assessed by 2D-TLC and was similar to that of the wild-type strain (Supplementary Figure 8). Thus, we suppose that the decreased sliding ability and biofilm formation of the espE mutant were not due to the alterations of the surface charge, hydrophobicity, or LOS biosynthesis.

Our results indicate that the deletion of espE, espF, espG, and espH resulted in extremely decreased sliding motility and biofilm formation. The incomplete restoration exhibited by complemented strains might be due to different expression levels of complemented genes carried by the plasmids, which was reported previously (Karnholz et al., 2006; Lin et al., 2006). 

M. marinum NTUH-M6885 could secrete EsxB protein and had a functional ESX-1 system. In addition, our data show that espE, espF, espG, and espH mutants accumulated EsxB protein in the cytosol and had reduced EsxB protein in the cell filtrates when compared with the wild-type strain (Supplementary Figure 6B). These results are consistent with those of a previous study that indicated that an M. marinum espE transposon mutant could not secrete EsxB protein and accumulated it in the cytosol (Carlsson et al., 2009). EsxB plays roles in cell membrane lysis and virulence (Unnikrishnan et al., 2017). Several previous studies showed that the secretion of the EssA-EssB heterodimer is co-dependent on the secretion of the EspA-EspC heterodimer, suggesting that the EssA-EssC heterodimer has an important function in ESX-1 secretion (Abdallah et al., 2007; Ates and Brosch, 2017; Wong, 2017). Additionally, the signal motifs of EspE and EspF were similar to those of EspA and EspC based on an analysis using the structural homology server PHYRE2 (Solomonson et al., 2015). Sliding motility and biofilm formation correlate with virulence in Mycobacterium spp. (Shorey and Sweet, 2008; Pang et al., 2013). Esp proteins regulate substrate export, which might be involved in sliding motility and biofilm formation.

The ESX-1 system is one of the major groups of T7SS (Bottai et al., 2011; Korotkova et al., 2014) and its role in pathogenesis has been reported in M. tuberculosis (Unnikrishnan et al., 2017; Wong, 2017) and M. marinum (Gao et al., 2004; Cardenal-Munoz et al., 2017; Unnikrishnan et al., 2017). Previous studies reported that espG and espH affect M. marinum virulence by influencing cytology, cytotoxicity, growth in macrophages, and spreading (Gao et al., 2004; McLaughlin et al., 2007). Bottai et al. (2011) indicated that espF and espG1 were associated with virulence during M. tuberculosis infection (Brodin et al., 2006) and M. tuberculosis can survive in bone marrow-derived macrophages. The present study is the first study to demonstrate the contribution of the ESX-1 system to sliding motility and biofilm formation in M. marinum.

In summary, we demonstrated that espE, espF, espG, and espH genes are critical for sliding motility and biofilm formation in M. marinum. These genes, which are located in the T7SS ESX-1 operon, are important virulence factors in M. marinum.

**AUTHOR CONTRIBUTIONS**

T-LL and J-TW designed the research, discussed the analysis, and revised the paper. L-YL and Y-YC prepared materials and
ACKNOWLEDGMENTS

We thank Dr. Feng-Ling Yang and Dr. Shih-Hsiung Wu (Institute of Biological Chemistry, Academia Sinica, Taipei, Taiwan) for their kind help with the lipid extraction.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.01160/full#supplementary-material
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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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