Insertion Mutation of *MSMEG_0392* Play an Important Role in Resistance of *M. smegmatis* to Mycobacteriophage SWU1

Zhen Zhang^{1,2}, Zhulan Yang³, Junfeng Zhen¹, Xiaohong Xiang⁴, Pu Liao¹, Jianping Xie²

¹Department of Clinical Laboratory, Chongqing General Hospital, University of Chinese Academy of Sciences, Chongqing, People's Republic of China;

²Institute of Modern Biopharmaceuticals, State Key Laboratory Breeding Base of Three Gorges Eco-Environment and Bioresources, Eco-Environment Key Laboratory of the Three Gorges Reservoir Region, Ministry of Education, School of Life Sciences, Southwest University, Chongqing, 400715, People's Republic of China; ³Department of Clinical Laboratory, Southwest Hospital, Army Medical University, Chongqing, People's Republic of China;

⁴School of Pharmacy, Chongqing Medical and Pharmaceutical College, Chongqing, People's Republic of China

Correspondence: Jianping Xie; Pu Liao, Tel/Fax +8623-68367108, Email georgex@swu.edu.cn; liaopu@sina.com

Purpose: Phage is a new choice for the treatment of multi-drug-resistant bacteria, and phage resistance is also an issue of concern. SWU1 is a mycobacteriophage, and the mechanism of its resistance remain poorly understood.

Methods: The mutant strains which were stably resistant to SWU1 were screened by transposon mutation library. The stage of phage resistance was observed by transmission electron microscope (TEM). The insertion site of transposon was identified by thermal asymmetric interlaced PCR (TAIL-PCR). The possible relationship between insertion site and phage resistance was verified by gene knockout technique. The fatty acid composition of bacterial cell wall was analyzed by Gas Chromatography-Mass Spectrometer (GC-MS). Through the amplification and sequencing of target genes and gene complement techniques to find the mechanism of SWU1 resistance.

Results: The transposon mutant M12 which was stably resistant to mycobacteriophage SWU1 was successfully screened. It was confirmed that resistance occurred in the adsorption stage of bacteriophage. It was verified that the insertion site of the transposon was located in the *MSMEG_3705* gene, but after knocking out the gene in the wild type *M. smegmatis* mc2 155, the resistance of the knockout strain to SWU1 was not observed. Through the amplification and sequencing of the target gene *MSMEG_0392*, it was found that there was an adenine insertion mutation at position 817. After complementing *MSMEG_0392* in M12, it was found that M12 returned to sensitivity to SWU1.

Conclusion: We confirmed that the resistance of M12 to SWU1 was related to the functional inactivation of *MSMEG_0392* and this phenomenon may be caused by the change of cell wall of *M. smegmatis*.

Keywords: phage resistance *M. smegmatis*, mycobacteriophage, SWU1, insertion mutation

Introduction

Mycobacteria have been classified into various types based on evolution and different genomic distributions, this mainly includes *Mycobacterium tuberculosis complex* (MTBC), *Mycobacterium leprae* and various environmental mycobacteria, also known as non-tuberculosis mycobacteria (NTM), they have varying degrees of pathogenicity and virulence in the clinic¹ and can cause different degrees of infection in patients. *Mycobacterium smegmatis* is a fast-growing bacterium that is generally not pathogenic in the clinic, its genome has high homology with *Mycobacterium tuberculosis*, and it is an ideal model strain to study mycobacterial infection. The cell wall of *Mycobacterium* consists of a variety of biological macromolecules, including peptidoglycans, arabinogalactans, mycobacterium acids, lipids, etc., form a hydrophobic barrier for safety,²⁻⁴ among which Lipids play an important role in the pathogenicity and virulence of Mycobacterium.^{5,6}

Bacteriophages are viruses that infect bacterial hosts and are the most abundant microorganisms in the biosphere. Bacteriophage is a potential tool in the treatment of bacterial infection, rapid detection of pathogens, bacterial typing,

control of bacterial contamination in food, treatment of environmental pollution, control of pathogen transmission and molecular biology research.^{7–14} It has been reported that bacteriophages are used to treat *Mycobacterium* infections, for example, in 2019, the United States researchers clinically improved one case of cystic fibrosis with disseminated mycobacterial infection by intravenous injection of three mycobacterial bacteriophages.¹⁵ In addition, bacteriophages also have been reported for the treatment of Carbapenem-Resistant *Klebsiella pneumoniae*,^{16–19} multidrug-resistant *Acinetobacter baumannii*,²⁰ pan-drug resistant *Pseudomonas aeruginosa* et al.²¹ But we cannot be too blindly optimistic, because we have to face the reality that bacteria are resistant to bacteriophages.^{22–25}

Currently, phage resistance has been found in a variety of bacteria, with varying mechanisms of resistance, which may occur during each process of phage invasion of the host.^{26,27} Among them, most of the literatures reported that it occurs in the adsorption stage. For example, *Klebsiella pneumoniae* was recently reported to affect phage adsorption through multiple evolutions, causing phage resistance to occur.²² Multiple components of the bacterial cell wall can serve as receptors for the adsorption of bacteriophages, such as Glycopeptidolipids (GPLs). GPLs is a major glycolipid present in the cell wall of various *Mycobacterium*. It plays an important role in bacterial drug resistance and host-pathogen interaction, and also in phage resistance of Mycobacteria.²⁸ Glycosyltransferases are widely found in mycobacterial cells. These enzymes transfer glycosyl onto a range of substrates and participate in the synthesis of GPLs.^{29–31}

SWU1 is a virus that infects mycobacterial hosts.³² In this study, we found that a *M. smegmatis* Tn5 transposon mutant strain M12 was resistant to SWU1, and further explored the possible mechanism of phage resistance of *M. smegmatis*.

Materials and Methods

Culture Conditions of Strains and Phages

Mycobacteriophage SWU1 was isolated from soil from Sichuan Province.³² *M. smegmatis* mc² 155 was cultured in Middlebrook 7H9 medium with 0.05% Tween 80. All the cultures were grown at 37°C. Phage SWU1 was grown as described previously.³² The primers used for this test are shown in Table 1

Screening of SWU1 Resistant Mutant Strain

Using Tn5 transposon, we constructed a mutant library containing about 300 transposon mutants.³³ For the screening of the library clones, the mutants in the library were inoculated individually into a 96-well plate containing 200μL 7H9 liquid medium (without Tween80) and 20μg/mL Kanamycin. After incubation for 48 h, the fresh culture was inoculated into the new 96-well plate with the same culture medium according to the ratio of 1: 100 (cells volume: medium volume), and cultured again for 48 hours. Then the 5μL mutant suspension was dropped into a plate containing 1×10¹⁰ PFU/mL SWU1 bacteriophage, and nine mutants were tested on each plate. After screening the mutants that may be resistant to SWU1, we further verified the resistance performance. The method is as follows: the dilution range of SWU1 bacteriophage was from 0 to 10⁻⁵, and 5μL of the diluted phages was dripped into the 7H9 Agar plate containing *M. smegmatis* mc² 155 and transposon mutants, respectively. Plaque formation was observed after the plate was placed in a 37 °C incubator and cultured for 24 hours.

Electroporation of SWU1 Genome into M12 and *M. Smegmatis* mc² 155

M12 and *M. smegmatis* mc² 155 were cultured to logarithmic growth phase to prepare competent cells. The 5μL SWU1 genome (total 50ng) was added to 400μL M12 and *M. smegmatis* mc² 155 competent cells, mixed well and kept in an ice bath for 30 min. After that, the mixture was transferred into a precooled electric cuvette, and 2500V was used for electroconversion. Subsequently, samples were placed on ice for 10 min, 1mL 7H9 medium was added and revived overnight in 110 rpm shaker at 37 °C. Resuspended bacteria were added to 4mL 7H9 medium (containing 0.35% agarose) at 40–50°C, mixed, and poured into 7H9 solid plates. The plate was cultured in an incubator at 37 °C for 48 hours.

Table I Primers Used in This Study

Name	Primer Sequence	Function
LAD-1	5'ACGATGGACTCCAGAGCGGCCGC(G/C/A)N(G/C/A)NNNGGAA-3'	Identification of transposon insertion sites
SP-1	5'-TGATGCTCGATGAGTTTTTCTAATCAGAAT-3'	Identification of transposon insertion sites
SP-2	5'ACGATGGACTCCAGAGTCCGGCCTGGTTAATTGGTTGTAACACTGGCAGAGC-3'	Identification of transposon insertion sites
SP-3	5'-ATCAGATCACGCATCTTCCCGACAACGCAGA-3'	Identification of transposon insertion sites
AC	5'-ACGATGGACTCCAGAG-3'	Identification of transposon insertion sites
3705-1	5'-AGGGATCCATGTCAGCACACAGG-3'	Verification of the transposon insertion sites
3705-2	5'-GCGATCGATTGAGCGAGCCTCTTC-3'	Verification of the transposon insertion sites
KOP1	5'- <u>AAGCTT</u> GCGTGGTCGAACTGTT-3'	MSMEG_3705 upstream 600bp amplification
KOP2	5'-CCTCGCCGCGAGCGCAGAAC <u>AGATCT</u> CAGCGTCTCCTCGTCGGGAT-3'	MSMEG_3705 upstream 600bp amplification
KOP3	5'- <u>AGATCT</u> GTTCTGCGCTCGCGCGAGG-3'	MSMEG_3705 downstream 600bp amplification
KOP4	5'- <u>AAGCTT</u> GCGCCCGCACCAGGCCT-3'	MSMEG_3705 downstream 600bp amplification
KOCP1	5'-AAGGCCGTACGCGCATT-3'	Confirmation of MSMEG_3705 replacement with dif sequence
KOCP2	5'-TACGTGGCAGCCACCTCA-3'	Confirmation of MSMEG_3705 replacement with dif sequence
0392P1	5'-CCGGATCCATGTTACGCTCCACG-3'	Used to amplify MSMEG_0392 gene
0392P2	5'-CCGAATTCTCACATCGCCGCTTGGC-3'	Used to amplify MSMEG_0392 gene

Analysis of Bacteriophage Adsorption by TEM

M. smegmatis mc² 155 and M12 were cultured to logarithmic growth phase, then the cultures were collected and adjusted to an OD₆₀₀ of 1.0. Five hundred-microliter of the adjusted samples were taken into EP tube, add SWU1, make the multiplicity of infection (MOI) 0.1 and incubated for 30min at 37°C. 20μL of the incubated sample was dropped onto the copper grid, pipetted repeatedly to make it adhere to the copper grid, filter paper was used to absorb excess liquid from the sides, dried and then observed by FEI TECNAI10 transmission electron microscope for the adsorption of SWU1 to both strains.

Identification of Transposon Insertion Sites by TAIL-PCR

A modified TAIL-PCR was used to identify the insertion site of Tn5 transposon.³⁴ The brief steps as follows: primers are designed to create binding sites on the target sequence, and nested PCR is used to improve the specificity of the amplification products. After that, two rounds of high temperature and one round of low temperature alternating super PCR cycle were used to increase the concentration of specific products, thus reducing the proportion of non-specific products. Then the diluted second round PCR amplification products were used as the template of the third round PCR reaction. Through such three rounds of PCR, the specific sequence of transposon insertion site was obtained, and then the

specific sequence was sequenced and the loci of transposon insertion into the genome of *M. smegmatis* mc² 155 were found by comparing the homologous sequences in NCBI database. After that, we designed specific primers for the genes on the BLAST alignment, and verified the insertion site of the transposon by PCR and sequencing.

Construction of *MSMEG_3705* Knock-Out Strain

MSMEG_3705 were knocked out based on homologous recombination technology.³⁵ Experimental procedures refer to our published article.³⁶ The brief steps as follows: Two pairs of primers (KOP1/KOP2 and KOP3/KOP4) were used to amplify the upstream and downstream 600bp of *MSMEG_3705* respectively. The amplified fragment was treated and linked with hygromycin resistance gene fragment, and then transformed into DH5 α to recover the plasmid. Recombinant DNA was transferred into wild-type *M. smegmatis*, and the positive clones were screened with medium containing 50 mg/mL hygromycin. The *MSMEG_3705* knock-out strain without hygromycin resistance gene fragment was obtained by continuous culture. Finally, knock-out strains were verified by specific primers (KOCPI/KOCP2).

Detection of Sensitivity of Knock-Out Strain to SWU1

The knockout strain was cultured to the logarithmic phase while enriched for SWU1. The sensitivity of M12 to SWU1 was detected by Direct Spot Test method.³⁷ The steps as follows: resuspended bacteria were added to 4mL 7H9 medium (containing 0.35% agarose) at 40–50°C, mixed, and poured into 7H9 solid plates. SWU1 was diluted in a 10-fold concentration gradient, and 5 μ L of different concentration dilutions were dropped into the upper layer of double-layer 7H9 plate, respectively, after drying, put it into an incubator at 37°C for 24 hours.

Fatty Acids Extraction and GS/MS

The wild-type strain and M12 were inoculated in 7H9 liquid medium and cultured in 37°C with constant shaking (200 rpm) to logarithmic growth phase. The cells were collected by centrifugation and washed twice with PBS buffer. Saponification reaction: add 1.0mL reagent I (45g NaOH, 150mL methanol, 150mL distilled water) to the collected 100mg bacteria precipitation, seal the tube, shake it slightly in the boiling water bath for 5–10 s, let it boil for 5min, shake it violently for 5–10s, and then put it back into the boiling water bath to boil for 25 min. Methylation: add 2mL reagent II (325mL hydrochloric acid, 6.0N Standardized Solution and 275mL methanol) to the cooled tube and vortex gently, heating at 80°C for 10 min. Extraction: add 1.25mL reagent III (200mL hexane, 200mL methyl tert-butyl ether) to the cooled tube, invert gently for 10min, and remove the water phase (bottom layer of the test tube). Product cleaning: place approximately 3mL reagent IV (10.8 g NaOH is dissolved in 900mL water) was added to the organic phase of the test tube and turned over evenly for 5min. The organic phase was transferred to a new tube. After dehydration and concentration, methyl hexadecanoate was used as internal standard and 10 μ L sample was used for the Agilent 890A_5975C GC_MS detection.

Amplification, Sequencing and Comparison of *MSMEG_0392*

Two pairs of primers (0392P1 and 0392P2) were designed using Primer Premier 6 to amplify the *MSMEG_0392* sequences from M12 and *M. smegmatis* mc² 155 genome. The amplified products were sent to Beijing Genomics Institution (BGI) for sequencing, and the results were compared by BLAST.

Construction of M12_pNIT_ *MSMEG_0392*_Myc Complementary Strain

MSMEG_0392 gene was amplified by 0392P1 and 0392P2 primers from wild-type *M. smegmatis* mc² 155. The amplified products and pNIT_Myc were digested with *Bam*H I and *Eco*R I, respectively, and then ligated with T4 DNA ligase to form pNIT_ *MSMEG_0392*_Myc recombinant plasmid. The recombinant plasmid was transferred into M12 competent cells by electroporation to construct M12_pNIT_ *MSMEG_0392*_Myc complementary strain, and M12_pNIT_Myc was used as control.

Figure 1 Sensitivity of wild-type *Mycobacterium smegmatis* mc² 155 and M12 to phage SWU1. (A) wild-type *M. smegmatis* mc² 155 strain can form obvious plaque for SWU1. (B) M12 mutant strain cannot form plaque.

Results

Resistance of M12 to SWU1

We screened the mutants from the *M. smegmatis* mc² 155 transposon library,³³ and the results of the first round of screening showed that six strains (M321, M346, M305, M310, M12 and M317) preliminary resistance to SWU1. Subsequently, we carried out a number of experiments to validate six strains again by using Direct Spot Test method, and the results confirmed that only M12 was stably resist to SWU1 (Figure 1).

Resistance of M12 to SWU1 Occurs in the Stage of Adsorption

To determine at which stage the resistance of M12 to SWU1 occurred, we transferred the SWU1 genome into M12 and *M. smegmatis* mc² 155 competent cells respectively. The results showed that clear plaque could be seen on the plates of *M. smegmatis* mc² 155 and M12 strain (Figure 2). This suggests that the mutation present in M12 does not affect the replication, transcription, assembly and release of SWU1. The resistance of M12 to SWU1 likely occurs in the adsorption or injection of phage SWU1. Then, we observed the adsorption of SWU1 on *M. smegmatis* by TEM. The results of TEM showed that compared with the wild-type strain, fewer phages accumulated around the M12 mutant strain (Figure 3). This indicates that the mutation of M12 may lead to the weakening of phage adsorption capacity.

Figure 2 Detection of M12 resistance to SWU1. (A) As a blank control. (B) M12, and (C) *M. smegmatis* mc² 155 does not affect the replication of SWU1. Red arrows indicate plaques of phages.

Figure 3 M12 mutant strain affects SWU1 adsorption to mycobacteria. **(A and B)** Wild-type *M. smegmatis* mc² 155 can adsorb SWU1 normally. **(C and D)** The adsorption capacity of M12 to SWU1 was significantly lower than that of *M. smegmatis* mc² 155. Red arrows indicate the location of phage presence.

Figure 4 The cell wall of M12 mutant was changed. Single colony phenotype of **(A)** M12 mutant and **(B)** wild-type *M. smegmatis* mc² 155 strains. **(C/D)** The fatty acid analysis results of **(C)** M12 mutant strains and **(D)** wild-type *M. smegmatis* mc² 155. Red boxes indicate a significant change in the abundance of components of the content. **(E)** Changes of fatty acid content in cell wall of M12 mutant compared with *Mycobacterium smegmatis* MC² 155.

The Cell Wall of M12 Mutant Was Changed

The resistance of M12 to SWU1 occurs at the adsorption stage, which makes us speculate that the cell wall of M12 mutant strain might change. Subsequently, we observed that the colony of M12 (Figure 4A) was smooth, shiny and neat compared with wild-type *M. smegmatis* mc² 155, while the surface of colony of wild-type *M. smegmatis* mc² 155 was rough, wrinkled and irregular (Figure 4B). This confirms our speculation that M12 resistance to phages may be associated with changes occurring in the mycobacterial cell wall. Next, we further analyzed the wild-type *M. smegmatis*

Figure 5 Transposon insertion site is located in the gene *MSMEG_3705*. **(A)** PCR amplification of *MSMEG_3705* gene by specific primers. Lane 1: Marker DL8000. Lane 2/3: *MSMEG_3705* amplification product using M12 as template. Lane 4/5: *MSMEG_3705* amplification using *M. smegmatis* mc² 155 as template. Lane 6: Marker DL2000. **(B)** Schematic diagram of insertion of Tn5 transposon into *M. smegmatis* mc² 155. The asterisk indicates the transposon Tn5 insertion site.

mc² 155 and M12 mutant strains cell wall composition changes. Using methyl nonadecanoate as the internal standard, we analyzed the fatty acid changes in different strains by gas chromatography-mass spectrometry (GC-MS). The results showed that there was no significant difference in the type of fatty acids in the cell wall of M12 mutant strain (Figure 4C) and wild-type *M. smegmatis* mc² 155 (Figure 4D), but as point out in the figure, the abundance of some fatty acids changed significantly. Especially c14:1W5C, M12 content is 58.44 times that of wild-type *M. smegmatis* mc² 155 (Figure 4E). It is consistent with our previous conclusion that the cell wall components of M12 mutant strains changed, which led to its resistance to phages.

Transposon Insertion Site is Located in the Gene *MSMEG_3705*

To determine the specific location of M12 in the *M. smegmatis* mc² 155 genome, we used the modified TAIL-PCR method to identify transposon insertion site. By PCR reaction, sequencing, sequence alignment, it was found that the transposon insertion site was located in *M. smegmatis* mc² 155 *MSMEG_3705* gene, which encodes a selective major facilitator superfamily efflux pump with multiple roles.³⁶ We further designed specific primers at both ends of the *MSMEG_3705* gene and found that the amplification product using the M12 genome as the template was about 1200bp larger (the size of Tn5 sequence is 1221bp) than that using the wild-type *M. smegmatis* mc² 155 genome as the template (Figure 5A). The amplification products were sent to BGI for sequencing, and the results showed that the Tn5 transposon was inserted into the 1064bp of the gene *MSMEG_3705* (Figure 5B).

M12 Resistance to SWU1 Did Not Correlate with the Inactivation of *MSMEG_3705*

To determine whether the phenotype of phage resistance of M12 is caused by the loss of the function of *MSMEG_3705*, we constructed the knock-out strain of *MSMEG_3705* by homologous recombination. After the knock-out strain was successfully constructed, we tested the sensitivity of the *M. smegmatis* mc² 155 Δ *MSMEG_3705*, M12 and wild-type *M. smegmatis* mc² 155 to SWU1. The result is not that like the mutant strain, the Δ *MSMEG_3705* strain was sensitive to SWU1 (Figure 6). This implies that transposon insertion mutations may affect the functional exercise of other genes.

Figure 6 Sensitivity detection of different strains to SWU1. **(A)** *M. smegmatis* mc² 155 and **(B)** *M. smegmatis* mc²155 Δ *MSMEG_3705* was sensitive to SWU1. **(C)** M12 mutant strain cannot form plaque.

	780	790	800	810	820	830	840	850	860
M12 <i>MSMEG_0392</i>	TCGAGGATGCGCAACGGCGCGAACTCGGATTGCCACGGACGAA	GGTTCGTCCTCCCGCGGAGGATCGCCGAACGCGGGTCACTCGAGAT							
WT <i>MSMEG_0392</i>	TCGAGGATGCGCAACGGCGCGAACTCGGATTGCCACGGACGAA	GGTTCGTCCTCCCGCGGAGGATCGCCGAACGCGGGTCACTCGAGAT							
Standard sequence	TCGAGGATGCGCAACGGCGCGAACTCGGATTGCCACGGACGAA	GGTTCGTCCTCCCGCGGAGGATCGCCGAACGCGGGTCACTCGAGAT							
	tcgaggatgcgcaacggcgcgaaactcggattgccacggacgaa	gggtccgtctcccgcgaggatcgccgaacgcggggtcactcgagat							

Figure 7 Sequence comparison of *MSMEG_0392* products amplified from M12 and *M. smegmatis* mc² 155 as templates.

Figure 8 The sensitivity of M12_pNIT_0392 to SWU1 was detected by Direct Spot Test. (A) *M. smegmatis* mc² 155 and (B) M12_pNIT_0392 can form obvious plaque for SWU1. (C) M12 mutant strain and (D) M12_pNIT_Myc cannot form plaque.

MSMEG_0392 Insertion Mutation Causes M12 Resistance to SWU1

It has been reported that *MSMEG_0392* encodes a glycosyltransferase, which can affect the synthesis of *M. smegmatis* cell wall glycopeptidolipids³⁸ and plays a key role in *M. smegmatis* resistance to phage I3³⁹ and phage Weirido19ES.⁴⁰ Therefore, we designed primers to amplify *MSMEG_0392* and sent the amplified product to BGI for sequencing. The results showed that *MSMEG_0392* gene amplified fragment using M12 genome as a template had an Adenine insertion mutation at position 817 (Figure 7). Therefore, we have reason to speculate that *MSMEG_0392* plays a key role in M12 resistance to SWU1. Then, we complemented the *MSMEG_0392* gene into M12 mutant strain genome, and the further experimental results showed that the complementary strain was sensitive to SWU1 (Figure 8). These results demonstrated that *MSMEG_0392* gene play an important role in M12 resistance to SWU1.

Discussion

Phage infection of the host can be divided into different stages, such as adsorption, DNA injection, replication, transcription and translation, assembly of progeny phage and host cleavage, release of mature phage.⁴¹ Host interfere with any steps in the phage lysis cycle can lead to the production of phage resistance phenotype. Current research results show that the host can resistance bacteriophage infection through at least five mechanisms.⁴² It includes adsorption inhibition (including change the conformation of the receptor, loss or camouflage),^{43,44} DNA injection inhibition (superinfection exclusion system),⁴⁵ restriction modification system,⁴⁶ CRISPR mechanism,⁴⁷ abortion infection mechanism et al.⁴⁸ Among them, adsorption inhibition is the most common mechanism of host resistance to bacteriophages. A large number of articles have confirmed that the phage receptors are located in the cell wall or cell membrane of bacteria. Changes in bacterial cell wall or cell membrane components not only alter the morphology of single colony, but also cause a phenotype of phage resistance. The sooth type of *M. abscess* makes it impossible to be effectively killed by phages.⁴⁹

Glycosyltransferases are widely found in mycobacterial cells, and these enzymes transfer glycosyl onto a range of substrates and participate in the synthesis of GPLs. In addition, it also plays an important role in bacterial drug resistance and host-pathogen interaction and is involved in the phage resistance of *Mycobacteria*. We confirmed that there is an insertion mutation of adenine at position 817 of *MSMEG_0392* (1515 bp, encoding glycosyltransferase from 504 amino acids), which results in frame shift mutations, thus affecting the synthesis of GPLs in *Mycobacterium smegmatis* cell walls, resulted in the appearance of resistance phenotype of *M. smegmatis* to SWU1. Similarly, *MSMEG_0392* as glycosyltransferase also plays an important role in I3 phage resistance, but the functions of phage I3³⁹ and SWU1³² phage are not exactly the same, which seems to tell us that the change of *MSMEG_0392* or important components of *Mycobacterium* cell wall will directly affect phage resistance, and *MSMEG_0392* may be an important target of phage therapy. Of course, we do not know whether there are other factors causing the resistance phenotype of M12 to phage SWU1. Further genome-wide wild sequencing may find some other valuable factors. As far as we know, this is the first time to elucidate the mechanism of resistance of *M. smegmatis* to mycobacteriophage SWU1, this will provide us with a better understanding of the use of phages as therapeutic tools.

Conclusion

In this study, we screened the M12 mutant which was stably resistant to SWU1 from a transposon mutant library and confirmed that the transposon insertion mutation is located in the *MSMEG_3705* gene, but we did not find any evidence that phage resistance was directly related to the inactivation of the *MSMEG_3705*. Through a literature survey, *MSMEG_0392* was found to play a key role in *M. smegmatis* resistance to bacteriophage, so we examined the gene sequence of *MSMEG_0392* in M12. It was found that this gene had an insertion of adenine at position 817, which resulted in *MSMEG_0392* unable to function as a glycosyltransferase, and affected the biosynthesis of GPLs, thus affecting the adsorption of SWU1 to *M. smegmatis*, causing M12 resistance to SWU1phage. This suggests that the adenine site at position 817 of *MSMEG_0392* is a key site, able to affect its functional role, and responsible for the occurrence of resistance to phage.

Acknowledgments

This work was funded by innovation projects of Chongqing General Hospital (Grant No. 2016MSXM28), The general program of Chongqing Science and Technology Commission (Grant No. cstc2018jcyjAX0667).

Disclosure

The authors report no conflicts of interest in this work.

References

1. Rahman MM, Rahim MR, Khaled A, Nasir TA, Nasrin F, Hasan MA. Molecular detection and differentiation of mycobacterium tuberculosis complex and non-tuberculous mycobacterium in the clinical specimens by real time PCR. *Mymensingh Med J*. 2017;26(3):614–620.

2. Chen S, Teng T, Wen S, Zhang T, Huang H. The aceE involves in mycolic acid synthesis and biofilm formation in *Mycobacterium smegmatis*. *BMC Microbiol*. 2020;20(1):259. doi:10.1186/s12866-020-01940-2
3. Chatterjee D. The mycobacterial cell wall: structure, biosynthesis and sites of drug action. *Curr Opin Chem Biol*. 1997;1(4):579–588. doi:10.1016/S1367-5931(97)80055-5
4. Brennan PJ. Structure, function, and biogenesis of the cell wall of *Mycobacterium tuberculosis*. *Tuberculosis*. 2003;83(1–3):91–97. doi:10.1016/S1472-9792(02)00089-6
5. Wang Z, Schwab U, Rhoades E, Chess PR, Russell DG, Notter RH. Peripheral cell wall lipids of *Mycobacterium tuberculosis* are inhibitory to surfactant function. *Tuberculosis*. 2008;88(3):178–186. doi:10.1016/j.tube.2007.11.003
6. Llorens-Fons M, Perez-Trujillo M, Julian E, et al. Trehalose polyphosphates, external cell wall lipids in *Mycobacterium abscessus*, are associated with the formation of clumps with cording morphology, which have been associated with virulence. *Front Microbiol*. 2017;8:1402. doi:10.3389/fmicb.2017.01402
7. Akinyemi KO, Philipp W, Beyer W, Böhm R. Application of phage typing and pulsed-field gel electrophoresis to analyse *Salmonella enterica* isolates from a suspected outbreak in Lagos, Nigeria. *J Infect Dev Ctries*. 2010;4(12):828–833. doi:10.3855/jidc.744
8. Crunkhorn S. Phage therapy for *Mycobacterium abscessus*. *Nat Rev Drug Discov*. 2019;18(7):500.
9. El-Shibiny A, El-Sahhar S, Adel M. Phage applications for improving food safety and infection control in Egypt. *J Appl Microbiol*. 2017;123(2):556–567. doi:10.1111/jam.13500
10. Hashemi Shahraki A, Mirsaedi M. Phage therapy for *Mycobacterium abscessus* and strategies to improve outcomes. *Microorganisms*. 2021;9(3):596. doi:10.3390/microorganisms9030596
11. Hemvani N, Patidar V, Chitnis DS. A simple and economical in-house phage technique for the rapid detection of rifampin, isoniazid, ethambutol, streptomycin, and ciprofloxacin drug resistance in *Mycobacterium tuberculosis*, directly on decontaminated sputum samples. *Int J Infect Dis*. 2012;16(5):e332–e336. doi:10.1016/j.ijid.2011.12.016
12. Nelson EJ, Chowdhury A, Flynn J, et al. Transmission of *Vibrio cholerae* is antagonized by lytic phage and entry into the aquatic environment. *PLoS Pathog*. 2008;4(10):e1000187. doi:10.1371/journal.ppat.1000187
13. Senhaji-Kacha A, Esteban J, Garcia-Quintanilla M. Considerations for phage therapy against *Mycobacterium abscessus*. *Front Microbiol*. 2020;11:609017. doi:10.3389/fmicb.2020.609017
14. Vikram A, Tokman JI, Woolston J, Sulakvelidze A. Phage biocontrol improves food safety by significantly reducing the level and prevalence of *Escherichia coli* O157: H7 in various foods. *J Food Prot*. 2020;83(4):668–676. doi:10.4315/0362-028X.JFP-19-433
15. Dedrick RM, Guerrero-Bustamante CA, Garlena RA, et al. Engineered bacteriophages for treatment of a patient with a disseminated drug-resistant *Mycobacterium abscessus*. *Nat Med*. 2019;25(5):730–733. doi:10.1038/s41591-019-0437-z
16. Wang Z, Cai R, Wang G, et al. Combination therapy of phage vB_KpnM_P-KP2 and gentamicin combats acute pneumonia caused by K47 serotype *Klebsiella pneumoniae*. *Front Microbiol*. 2021;12:674068. doi:10.3389/fmicb.2021.674068
17. Cano EJ, Cafilisch KM, Bollyky PL, et al. Phage therapy for limb-threatening prosthetic knee *Klebsiella pneumoniae* infection: case report and in vitro characterization of anti-biofilm activity. *Clin Infect Dis*. 2021;73(1):e144–e151. doi:10.1093/cid/ciaa705
18. Anand T, Virmani N, Kumar S, et al. Phage therapy for treatment of virulent *Klebsiella pneumoniae* infection in a mouse model. *J Glob Antimicrob Resist*. 2020;21:34–41. doi:10.1016/j.jgar.2019.09.018
19. Hung CH, Kuo CF, Wang CH, Wu CM, Tsao N. Experimental phage therapy in treating *Klebsiella pneumoniae*-mediated liver abscesses and bacteremia in mice. *Antimicrob Agents Chemother*. 2011;55(4):1358–1365. doi:10.1128/AAC.01123-10
20. Bagińska N, Cieślak M, Górski A, Jończyk-Matysiak E. The role of antibiotic resistant *A. baumannii* in the Pathogenesis of urinary tract infection and the potential of its treatment with the use of bacteriophage therapy. *Antibiotics*. 2021;10(3):281. doi:10.3390/antibiotics10030281
21. Engeman E, Freyberger HR, Corey BW, et al. Synergistic killing and re-sensitization of *Pseudomonas aeruginosa* to antibiotics by phage-antibiotic combination treatment. *Pharmaceuticals*. 2021;14(3):184. doi:10.3390/ph14030184
22. Hesse S, Rajaure M, Wall E, et al. Phage resistance in multidrug-resistant *Klebsiella pneumoniae* ST258 evolves via diverse mutations that culminate in impaired adsorption. *mBio*. 2020;11(1):e02530–e02519. doi:10.1128/mBio.02530-19
23. Tan D, Zhang Y, Qin J, et al. A frameshift mutation in wcaJ associated with phage resistance in *Klebsiella pneumoniae*. *Microorganisms*. 2020;8(3):378. doi:10.3390/microorganisms8030378
24. Henrici De Angelis L, Poerio N, Di Pilato V, et al. Phage resistance is associated with decreased virulence in KPC-producing *Klebsiella pneumoniae* of the Clonal Group 258 Clade II Lineage. *Microorganisms*. 2021;9(4):762. doi:10.3390/microorganisms9040762
25. Majkowska-Skronek G, Markwitz P, Sosnowska E, Lood C, Lavigne R, Drulis-Kawa Z. The evolutionary trade-offs in phage-resistant *Klebsiella pneumoniae* entail cross-phage sensitization and loss of multidrug resistance. *Environ Microbiol*. 2021;23(12):7723–7740. doi:10.1111/1462-2920.15476
26. Laanto E, Makela K, Hoikkala V, Ravaniti JJ, Sundberg LR. Adapting a phage to combat phage resistance. *Antibiotics*. 2020;9(6). doi:10.3390/antibiotics9060291
27. Broniewski JM, Meaden S, Paterson S, Buckling A, Westra ER. The effect of phage genetic diversity on bacterial resistance evolution. *ISME J*. 2020;14(3):828–836. doi:10.1038/s41396-019-0577-7
28. Bakli M, Karim L, Mokhtari-Soulmane N, Merzouk H, Vincent F. Biochemical characterization of a glycosyltransferase Gtf3 from *Mycobacterium smegmatis*: a case study of improved protein solubilization. *3 Biotech*. 2020;10(10):436. doi:10.1007/s13205-020-02431-x
29. Deshayes C, Laval F, Montrozier H, Daffe M, Etienne G, Reyat JM. A glycosyltransferase involved in biosynthesis of triglycosylated glycopeptidolipids in *Mycobacterium smegmatis*: impact on surface properties. *J Bacteriol*. 2005;187(21):7283–7291. doi:10.1128/JB.187.21.7283-7291.2005
30. Malm S, Walter K, Engel R, et al. In vitro and in vivo characterization of a *Mycobacterium tuberculosis* mutant deficient in glycosyltransferase Rv1500. *Int J Med Microbiol*. 2008;298(7–8):645–655. doi:10.1016/j.ijmm.2008.03.010
31. Sarkar D, Sidhu M, Singh A, et al. Identification of a glycosyltransferase from *Mycobacterium marinum* involved in addition of a caryophyllose moiety in lipooligosaccharides. *J Bacteriol*. 2011;193(9):2336–2340. doi:10.1128/JB.00065-11
32. Fan X, Teng T, Wang H, Xie J. Biology of a novel mycobacteriophage, SWU1, isolated from Chinese soil as revealed by genomic characteristics. *J Virol*. 2012;86(18):10230–10231. doi:10.1128/JVI.01568-12

33. Du Q, Long Q, Mao J, Fu T, Duan X, Xie J. Characterization of a novel mutation in the overlap of tlyA and ppnK involved in capreomycin resistance in Mycobacterium. *IUBMB Life*. 2014;66(6):405–414. doi:10.1002/iub.1277
34. Liu YG, Chen Y. High-efficiency thermal asymmetric interlaced PCR for amplification of unknown flanking sequences. *Biotechniques*. 2007;43(5):649–650, 652, 654 passim. doi:10.2144/000112601
35. Cascioferro A, Boldrin F, Serafini A, Provvedi R, Palù G, Manganelli R. Xer site-specific recombination, an efficient tool to introduce unmarked deletions into mycobacteria. *Appl Environ Microbiol*. 2010;76(15):5312–5316. doi:10.1128/AEM.00382-10
36. Zhang Z, Wang R, Xie J. Mycobacterium smegmatis MSMEG_3705 encodes a selective major facilitator superfamily efflux pump with multiple roles. *Curr Microbiol*. 2015;70(6):801–809. doi:10.1007/s00284-015-0783-0
37. Haines MEK, Hodges FE, Nale JY, et al. Analysis of selection methods to develop novel phage therapy cocktails against antimicrobial resistant clinical isolates of bacteria. *Front Microbiol*. 2021;12:613529. doi:10.3389/fmicb.2021.613529
38. Miyamoto Y, Mukai T, Nakata N, et al. Identification and characterization of the genes involved in glycosylation pathways of mycobacterial glycopeptidolipid biosynthesis. *J Bacteriol*. 2006;188(1):86–95. doi:10.1128/JB.188.1.86-95.2006
39. Chen J, Kriakov J, Singh A, Jacobs WR, Besra GS, Bhatt A. Defects in glycopeptidolipid biosynthesis confer phage I3 resistance in Mycobacterium smegmatis. *Microbiology*. 2009;155(Pt12):4050–4057. doi:10.1099/mic.0.033209-0
40. Suarez CA, Franceschelli JJ, Tasselli SE, Morbidoni HR. Weirido19ES is a novel singleton mycobacteriophage that selects for glycolipid deficient phage-resistant M. smegmatis mutants. *PLoS One*. 2020;15(5):e0231881. doi:10.1371/journal.pone.0231881
41. Espejo RT, Sinsheimer RL. The process of infection with bacteriophage phiX174. XXXIX. The structure of a DNA form with restricted binding of intercalating dyes observed during synthesis of phiX single-stranded DNA. *J Mol Biol*. 1976;102(4):723–741. doi:10.1016/0022-2836(76)90288-6
42. Zhang Z, Huang C, Pan W, Xie J. Intriguing arms race between phages and hosts and implications for better anti-infectives. *Crit Rev Eukaryot Gene Expr*. 2013;23(3):215–226. doi:10.1615/CritRevEukaryotGeneExpr.2013007250
43. Tükel C, Sanlibaba P, Özden B, Akcelik M. Identification of adsorption inhibition, restriction/modification and abortive infection type phage resistance systems in Lactococcus lactis strains. *Acta Biol Hung*. 2006;57(3):377–385. doi:10.1556/ABiol.57.2006.3.11
44. Nir-Paz R, Eugster MR, Zeiman E, Loessner MJ, Calendar R. Listeria monocytogenes tyrosine phosphatases affect wall teichoic acid composition and phage resistance. *FEMS Microbiol Lett*. 2012;326(2):151–160. doi:10.1111/j.1574-6968.2011.02445.x
45. Shi K, Oakland JT, Kurniawan F, Moeller NH, Banerjee S, Aihara H. Structural basis of superinfection exclusion by bacteriophage T4 Spackle. *Commun Biol*. 2020;3(1):691. doi:10.1038/s42003-020-01412-3
46. Dupuis ME, Villion M, Magadan AH, Moineau S. CRISPR-Cas and restriction-modification systems are compatible and increase phage resistance. *Nat Commun*. 2013;4:2087. doi:10.1038/ncomms3087
47. Broniewski JM, Chisnall MAW, Høyland-Kroghsbo NM, Buckling A, Westra ER. The effect of Quorum sensing inhibitors on the evolution of CRISPR-based phage immunity in Pseudomonas aeruginosa. *ISME J*. 2021;15(8):2465–2473. doi:10.1038/s41396-021-00946-6
48. Abedon ST. Phage “delay” towards enhancing bacterial escape from biofilms: a more comprehensive way of viewing resistance to bacteriophages. *AIMS Microbiol*. 2017;3(2):186–226. doi:10.3934/microbiol.2017.2.186
49. Dedrick RM, Smith BE, Garlena RA, et al. Mycobacterium abscessus strain morphotype determines phage susceptibility, the repertoire of therapeutically useful phages, and phage resistance. *mBio*. 2021;12(2). doi:10.1128/mBio.03431-20

Infection and Drug Resistance

Dovepress

Publish your work in this journal

Infection and Drug Resistance is an international, peer-reviewed open-access journal that focuses on the optimal treatment of infection (bacterial, fungal and viral) and the development and institution of preventive strategies to minimize the development and spread of resistance. The journal is specifically concerned with the epidemiology of antibiotic resistance and the mechanisms of resistance development and diffusion in both hospitals and the community. The manuscript management system is completely online and includes a very quick and fair peer-review system, which is all easy to use. Visit <http://www.dovepress.com/testimonials.php> to read real quotes from published authors.

Submit your manuscript here: <https://www.dovepress.com/infection-and-drug-resistance-journal>