THE CONTRIBUTION OF *rshA* MUTATIONS AND EFFLUX PUMPS IN TIGECYCLINE RESISTANCE IN *MYCOBACTEROIDES ABSCESSUS*

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ABSTRACT

THE CONTRIBUTION OF *rshA* MUTATIONS AND EFFLUX PUMPS IN TIGECYCLINE RESISTANCE IN *MYCOBACTEROIDES ABSCESSUS*

Tigecycline is an important antibiotic in treating patients with *Mycobacteroides abscessus* infections. Tigecycline resistance was previously associated with dysregulated stress response induced by the abnormal interaction between the SigH and RshA factors. Two laboratory-derived tigecycline-resistant mutants (CL5A and CL6A) which possessed mutations in the *rshA* gene and upregulated efflux pumps were studied, to have a better understanding on the role of *rshA* mutations and efflux pumps in tigecycline resistance in *M. abscessus*. Gene complementation studies confirmed that the *rshA* mutations in the mutants caused tigecycline resistance. The assumption that *rshA* mutations disrupt the interaction between RshA and SigH factors was tested using the Bacterial Adenylate Cyclase Two-Hybrid (BACTH) assay. The results showed that the RshA-SigH interaction in the *rshA* mutant was indeed slower than in the positive controls. RT-qPCR analyses on the mutants showed an upregulation of the *sigH* gene which indicated the release of SigH following the disrupted RshA-SigH interaction. Three efflux genes (*MAB_1395*, *MAB_1396* and *MAB_1299c*) were also found to be upregulated. This indicated that efflux pumps may contribute to tigecycline resistance in *M. absccessus*. However, overexpression of the three efflux genes did not result in any significant increase in tigecycline resistance. This suggested that the upregulation of efflux genes seen was a consequence of

a *rshA* mutation, but was not, on its own, the cause of tigecycline resistance in this mutant. This study demonstrated that *rshA* mutations in *M. abscessus* could disrupt the interaction between RshA and SigH, two proteins that are transcriptional regulators of physiological stress response in mycobacteria. The disrupted interaction is believed to lead to the overexpression of *sigH* which causes *M. abscessus* to be resistant against tigecycline. The mechanism which SigH causes tigecycline resistance has yet to be elucidated. The role of efflux pumps in tigecycline resistance in *M. abscessus* requires further exploration.

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APPROVAL SHEET

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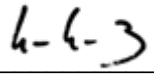

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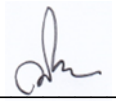

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SUBMISSION OF DISSERTATION

It is hereby certified that **AW KAR MEN** (ID No: **19UMM07057**) has completed this dissertation entitled "THE CONTRIBUTION OF *rshA* MUTATIONS AND EFFLUX PUMPS IN TIGECYCLINE RESISTANCE IN *MYCOBACTEROIDES ABSCESSUS*" under the supervision of Prof. Ngeow Yun Fong (Supervisor) from the Department of Pre-clinical Sciences, M. Kandiah Faculty of Medicine and Health Sciences, and Dr. Thaw Zin (Co-Supervisor) from the Department of Pre-clinical Sciences, M. Kandiah Faculty of Medicine and Health Sciences.

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DECLARATION

I hereby declare that the dissertation is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

(AW KAR MEN)

Date 10-06-2022

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LIST OF ABBREVIATIONS

Blastp	Protein Basic Local Alignment Search Tool
BACTH	Bacterial Adenylate Cyclase Two Hybrid system
CAMHA	Cation-adjusted Mueller-Hinton agar
CAMHB	Cation-adjusted Mueller-Hinton broth
CLSI	Clinical and Laboratory Standards Institute
EUCAST	European Committee on Antimicrobial Susceptibility Testing
M. abscessus	*Mycobacteroides abscessus*
MIC	Minimum inhibitory concentration
NCBI	National Center for Biotechnology Information
NTC	No-template control
NTM	Non-tuberculous mycobacteria
OD600	Optical density at 600 nm
RGM	Rapid-growing mycobacteria
RT-qPCR	Reverse-transcriptase real-time PCR
SOC	Super optimal broth with catabolite repression
UV	Ultraviolet
wt	Wild type

CHAPTER 1

INTRODUCTION

1.1 Background

Mycobacteroides abscessus, a nontuberculous rapid growing mycobacterium associated with skin, soft tissue and lung infections, is infamously known for its multidrug resistance phenotype (Brown-Elliott and Wallace, 2002; Nessar, et al., 2012). Tigecycline, a third-generation tetracycline with activity against bacteria resistant to earlier generation tetracyclines has shown efficacy against *M. abscessus* infections (Wallace, et al., 2014; Ferro, et al., 2016). Unfortunately, tigecycline resistance in clinical strains had been reported in Northeast Thailand (Ananta, et al., 2018). The emergence of clinically resistant strains had increased the urgency for more research to be done to prevent widespread tigecycline resistance in *M. abscessus*.

In Malaysia, researchers had discovered an association regarding tigecycline resistance with impaired interaction between the RshA, an anti-Sigma factor and SigH, a Sigma factor (Ng, et al., 2018; Lee, et al., 2021). In the absence of stress, RshA binds to SigH and prevents *sigH* and other stress-response related genes from being transcribed (Song, et al., 2006).

Interestingly, three efflux genes were also revealed to be upregulated in a tigecycline resistant *rshA* mutant (Ng, et al., 2018). This indicated that efflux genes may very likely contribute to tigecycline resistance seen in the mutant

Hence, this study was initiated to further elucidate the role of *rshA* mutations and efflux mechanisms in tigecycline resistance in *M. abscessus*. By gaining a deeper insight into the resistance mechanisms of *M. abscessus* this should hopefully lead to more appropriate antibiotic therapy and the development of new pharmaceutical strategies against *M. abscessus* infections.

1.2 Problem statement and hypothesis

While tigecycline may be effective in treating *M. abscessus* infections, the emergence of tigecycline resistance in *M. abscessus* may limit the lifespan of this rescue antibiotic, if this issue is left unsolved. Recently, *rshA* mutations were associated with tigecycline resistance and evidence showed that efflux pumps may be involved too. Unfortunately, the current knowledge on the mechanism of tigecycline resistance is limited.

Hence, this study was carried with the purpose to further investigate the role of *rshA* mutations and to determine whether efflux pumps do indeed play a part in tigecycline resistance in *M. abscessus.*

The research questions in this study were:

1. Do *rshA* mutations in CL5A and CL6A cause them to be resistant against tigecycline?
2. Is the efflux mechanism involved in causing *M. abscessus* to be resistant against tigecycline?

The hypothesis was:

Mutations in the *rshA* gene cause *M. abscessus* to be resistant to tigecycline by interrupting the RshA-SigH interaction leading to the overexpression of the *sigH* gene and upregulation of efflux pumps to increase the expulsion of tigecycline.

1.3 Objectives

The main objective for this study was to gain deeper insight into the mechanism of tigecycline resistance in *M. abscessus*.

The specific objectives in this study were:

1. to determine the causal relationship of the *rshA* mutation and tigecycline resistance in the tigecycline-resistant mutants, CL5A and CL6A using gene complementation
2. to study the RshA-SigH interaction in a tigecycline-resistant mutant using the Bacterial Adenylate Cyclase Two Hybrid (BACTH) system
3. to analyse *sigH* gene expression changes in tigecycline-resistant mutants using RT-qPCR

4. to investigate the causative role of the three efflux-encoding genes in tigecycline resistance in *M. abscesses* through a gene dosage study

The outcome of the study may contribute to the development of new and efficacious therapeutic strategies against *M. abscessus* infections.

CHAPTER 2

LITERATURE REVIEW

2.1 Nontuberculous and rapid growing Mycobacteria

The genus *Mycobacterium* encompasses a wide range of species (Gupta, Lo and Son, 2018), and according to the Runyon's classification, they could either be classified as rapid or slow growers (Griffith, et al., 2007; Medjahed, Gaillard and Reyrat, 2010). Rapid growing mycobacteria are generally defined as colonies visible on the culture media in less than a week (Hong, et al., 2003).

In contrast to *M. tuberculosis*, the obligate nontuberculous mycobacteria (NTM) (Medjahed, Gaillard and Reyrat, 2010), are environmental pathogens which share environmental niches such as soil and water with humans (van Ingen, et al., 2009). The persistent exposure allows the perfect opportunity for human infection (Honda, Virdi and Chan, 2018; Ratnatunga et al., 2020).

The epidemiologic data of NTM infections is unfortunately, scarce, as the reporting of NTM infections is not made compulsory in most countries (Prevots and Marras, 2015; To, et al., 2020). Nonetheless, the cases of NTM infections which are documented worldwide (Ratnatunga, et al., 2020) are reported to be increasing (Prevots and Marras, 2015; Stout, Koh and Yew, 2016) especially in developed countries in which tuberculosis infections are declining (To, et al., 2020).

Among the nontuberculous rapid growing mycobacteria, *M. abscessus* is one of the most malicious mycobacteria causing pulmonary infections, particularly in patients with underlying cystic fibrosis (Medjahed, Gaillard and Reyrat, 2010; Degiacomi, et al., 2019; Schiff, et al., 2019).

2.2 *Mycobacteroides abscessus* taxonomy

Since its discovery in the early 1950s (Moore and Frerichs, 1953), this abscess-causing pathogen has gone through several taxonomic changes following the advancements in the microbiological research field. Once considered to be the same species as *M. chelonei*, *M. abscessus* was later elevated into a species on its own with three other subspecies, *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *bolletii* and *M. abscessus* subsp. m*assiliense* (Brown-Elliott and Wallace, 2002).

Most recently, *M. abscessus* was reclassified as a new genus *Mycobacteroides* and is currently known as *Mycobacteroides abscessus* with the same three subspecies of *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *bolletii* and *M. abscessus* subsp. m*assiliense* (Gupta, Lo and Son, 2019).

2.3 *M. abscessus* environmental sources

Like the other NTM species, *M. abscessus* is an environmental mycobacterium (To, et al., 2020). It is also found in human drinking water sources, domestic and even hospital plumbing systems as *M. abscessus* can form biofilm and is resistant

to water treatment, disinfectants, antimicrobials as well as high temperatures (Falkinham, 2009; 2013; Thomson, et al., 2013; Degiacomi, et al., 2019; To, et al., 2020).

The persistence of mycobacteria and the sharing of habitation with the human population create the ideal setting for human infection (Degiacomi, et al., 2019; Ratnatunga, et al., 2020).

2.4 The underplayed player, *M. abscessus*

This environmental mycobacterium was initially considered to be a harmless pathogen with low virulence which causes self-limiting cutaneous infections (Moore and Frerichs, 1953).

However, the magnitude of *M. abscessus* could be severely underplayed in the past when it was still regarded to be the same species as *M. chelonei*. It was only until 1992, Kusunoki and Ezaki (1992) finally proposed for *M. abscessus* to be distinguished from *M. chelonei* and for it to be recognised as a species on its own. Hence, prior to 1992, the real culprit behind *M. chelonei* pulmonary infections could very likely be *M. abscessus*. While pulmonary infection is currently identified as a common clinical manifestation for *M. abscessus*, *M. chelonei* infrequently causes it (Ryan and Byrd, 2018). This discovery also highlighted that *M. abscessus* might have been excluded in literature or reports on pulmonary infections published prior to 1992 (Johansen, Herrmann and Kremer, 2020).

Nonetheless, since *M. abscessus* has been elevated to a species and later a novel genus on its own, this mycobacterium is now known to be one of the most fearful respiratory pathogens amid all the RGM, inflicting patients with underlying chronic lung diseases especially cystic fibrosis in hospitals worldwide (Degiacomi, et al., 2019).

2.5 Vulnerable groups for *M. abscessus* infections

M. abscessus is an opportunistic pathogen as it mostly troubles those who are immunosuppressed. This mycobacterium is widely feared among the patients with underlying lung diseases especially, cystic fibrosis (Esther, et al., 2010; Viviani, et al., 2016; Degiacomi, et al., 2019). This could be due to the association between cystic fibrosis transmembrane conductance regulator (CFTR) mutations and granuloma formation in patients with *M. abscessus* infections (Degiacomi, et al., 2019).

With improved microbiological techniques, it was shown that *M. abscessus* is transmissible between humans, in particular, cystic fibrosis patients (Bryant, et al., 2013). This discovery resulted in a revolutionary shift in the understanding of the transmission routes of *M. abscessus* (Yoshida, et al., 2018).

2.6 Clinical manifestations and outbreaks of *M. abscessus*

This nontuberculous rapid-growing mycobacterium has a broad spectrum of clinical diseases, ranging from skin and soft tissue infections (SSTIs) in the

healthy population to chronic pulmonary infections in those who are immunocompromised (Wongkitisophon, et al., 2011; Lee, et al., 2015). It can cause infections through compromised skin barrier via contact with contaminated earth or water (Falkinham, 2009; 2013). In healthy hosts, SSTIs are frequently associated with posttraumatic injuries and after cosmetic or surgical interventions in which contaminated medical devices or implants are used (Brown-Elliott and Wallace, 2002; Petrini, 2006; Furuya, et al., 2008; Wongkitisophon, et al., 2011; Sfeir, et al., 2018).

In vulnerable patients with underlying chronic lung diseases, such as cystic fibrosis (CF), bronchiectasis and previous history of pulmonary tuberculosis, *M. abscessus* infections are commonly manifested as progressive pulmonary lung infections with persistent symptoms which lead to declining lung functions associated with poor quality of life (Esther, et al., 2010; Lee et al., 2015).

M. abscessus has also been identified as an upcoming pathogen commonly associated with outbreaks in healthcare-settings (Lee, et al., 2015; Mougari, et al., 2016).

Although uncommon, there were reports of community outbreaks of *M. abscessus* infections, associated with wading pools (Dytoc, et al., 2005; Carter, et al., 2019). In South Korea, there was a report on a spontaneous outbreak of cutaneous infections post-acupuncture therapy (Song, et al., 2006).

In addition, there were case reports of peritonitis caused by *M. abscessus* in patients undergoing dialysis in Malaysia (Chin, et al., 2018) and Japan (Yoshimura, et al., 2018) as well as paediatric cases of *M. abscessus* causing chronic otitis media (Myojin, et al., 2018). *M. abscessus* could also be the aetiological agent of central nervous system and ocular infections (Griffith, et al., 2007; Nessar, et al., 2012; Lee, et al., 2015).

2.7 Diagnosis of *M. abscessus* infections

It is a daunting process to diagnose a patient with *M. abscessus* infections as the isolation of the strain alone is not sufficient. The differentiation between *M. abscessus* colonisation and true disease is very challenging. Thus, in order to diagnose an *M. abscessus* pulmonary infection, the patient needs to have clinical symptoms which correlate with radiologic evidence and fulfil the microbiologic criteria.

The American Thoracic Society/Infectious Disease Society of America has published a set of clinical guidelines in an endeavour to standardise and guide the diagnosis of *M. abscessus* pulmonary infections (Griffith, et al., 2007).

The most recent update includes the need for positive culture of respiratory specimen and molecular identification as the gold standard for the laboratory diagnosis of *M. abscessus* infection (Cortes, Nessar and Singh, 2010; Jones, et al., 2019).

2.8 *M. abscessus* treatment strategies

Owing to the multidrug resistant phenotype, *M. abscessus* infections are highly challenging to treat (Nessar, et al., 2012). The Clinical and Laboratory Standards Institute (CLSI) recommended antimicrobial susceptibility testing by determining the minimum inhibitory concentration (MIC) using broth microdilution assay (To, et al., 2020; Weng, et al., 2020). The susceptibility panel covers 10 antimicrobials such as amikacin, cefoxitin and linezolid (Griffith, et al., 2007). The incubation period for the assay was extended to 14 days to detect inducible macrolide resistance (Nie, et al., 2015; Shen, et al., 2018; Huang, Yu and Huang, 2020).

For pulmonary infections, in addition to the fulfilment of the American Thoracic Society/Infectious Diseases Society of America (ATS/IDSA) diagnostic criteria for NTM-lung disease, the initiation of treatment should also be supported by the course and severity of the disease as well as the patient's condition (Griffith, et al., 2007; Weng, et al., 2020).

Presently, the development of an effective treatment regimen is still ongoing (Weng, et al., 2020) . The American Thoracic Society and Infectious Disease Society of America had previously recommended a multidrug macrolide-based therapy with one or more parenteral antibiotics guided by antibiotic susceptibility test results (Griffith, et al., 2007; Novosad, et al., 2016). The treatment outcomes were sadly unfavourable.

Hence, a new treatment regimen with an initial phase of treatment for a minimum of 4-weeks which includes intravenous amikacin, imipenem and tigecycline with oral macrolide followed by a continuation treatment phase of inhaled amikacin and an oral macrolide alongside with up to three other recommended antibiotics, was proposed in the 2017 British Thoracic Society guidelines (Haworth, et al., 2017; Weng, et al., 2020).

The incorporation of tigecycline in the treatment regimen against *M. abscessus* infections had since demonstrated encouraging treatment outcomes (Wallace, et al., 2002; 2014; Ferro, et al., 2016).

2.9 Antimicrobial resistance

The discovery of antibiotics shifted the paradigm of treating infections, particularly in those who are severely ill. Penicillin, the first beta-lactam antibiotic to be discovered, marked the beginning of various antibiotic classes which were used for treating bacterial infections. Antibiotics are typically categorised based on their mechanisms of action (Table 2.1).

However, the routine and often inappropriate use of antibiotics had threatened their efficacy, making infections a challenge to treat (Munita and Arias, 2016). Initially, confidence was boosted as new antibiotics were developed to overcome resistance (Spellberg and Gilbert, 2014). Nonetheless, with time, antimicrobial resistance was on the rise again and the development of novel antibiotics began to dwindle (Spellberg and Gilbert, 2014).

As the situation worsened, the World Health Organization identified antibiotic resistance as one of the major threats to the public health (WHO, 2021). The absence of effective antibiotics makes invasive medical procedures such as surgery, even more risky than they already are.

Bacteria share the same ecological niche with the naturally produced antimicrobial compounds which have harmful effects on them. For survival, bacteria evolved to possess intrinsic forms of resistance against the antimicrobial compounds (Munita and Arias, 2016). Intrinsic antibiotic resistance usually includes efflux pumps, target inactivating or modifying enzymes or structures which act as barriers to antibiotic entry (Peterson and Kaur, 2018).

Resistance could also be acquired through genetic mutations or possession of genetic determinants via horizontal gene transfer (Blair, et al., 2015). The mutations typically occur in genes which encode the drug target and transporters (Reygaert, 2018).

The four main mechanisms of antibacterial resistance are summarised in Table 2.2.

Table 2.1: The general mechanisms of antibiotic action

Mechanisms of action	Example of antibiotics	Reference
Inhibit protein synthesis	Macrolides Aminoglycoside Tetracycline Linezolid	(Van Hoek, et al., 2011; Kapoor, Saigal and Elongavan, 2017)
Inhibit nucleic acid synthesis	Quinolones Rifampicin	
Inhibit cell wall synthesis	Beta-lactam	

Table 2.2: Mechanisms of antibiotic resistance

Mechanisms of resistance	Example of antibiotics	Reference
Changes in the cell wall permeability	Macrolides Aminoglycoside Tetracyclines Linezolid	(Dever and Dermody, 1991; Spellberg and Gilbert, 2014; Ventola, 2015; Reygaert, 2018)
Enzymatic degradation or inactivation of drug	Aminoglycoside Beta-lactams	
Modification to drug target	Beta-lactams Aminoglycoside Tetracyclines Macrolides	
Efflux system	Tetracyclines Quinolones	

2.10 Antimicrobial resistance mechanisms in *M. abscessus*

M. abscessus infections are a nightmare to clinicians as this mycobacterium is notoriously known to be resistant to most commonly available antibiotics (Schmalstieg, et al., 2012). Following the advancement in microbiological

techniques over the years, researchers were able to shed light on several resistant mechanisms which are responsible for the multidrug resistance trait in *M. abscessus*, particularly after its genome sequence was made available (Ripoll, et al., 2009).

This fearful phenotype which limits the selection of antibiotics is conferred by either the natural resistance or acquired resistance possessed by the mycobacterium (Nessar, et al., 2012). Drug susceptibility also varies among the three *M. abscessus* subspecies, and this further complicates the treatment strategies (Kim, et al., 2010a; Nessar, et al., 2012).

The intrinsic resistance mechanisms of *M. abscessus* are associated with the thick, waxy mycobacterial cell wall and efflux systems which decrease the intracellular drug concentration (Luthra, Rominski and Sander, 2018). The lipid-rich cell wall works in synergy with other natural mechanisms, rendering most drugs ineffective (Nessar, et al., 2012).

The multidrug resistance phenotype is also largely attributed to the drug or target modifying enzymes expressed by *M. abscessus* (Luthra, Rominski and Sander, 2018). The addition of acetyl and phosphate groups on aminoglycosides by *M. abscessus* enzymes makes it less vulnerable to this drug group (Ripoll, et al., 2009). Beta-lactamases could also result in resistance to beta-lactams (Ripoll, et al., 2009). Tetracycline resistance in *M. abscessus* is associated with monooxygenation by MabTetX (Rudra, et al., 2018).

While a combination of antibiotics is often administered to patients with *M. abscessus* infections, Pryjma, et al. (2017) suggested otherwise as the study documented that amikacin and clarithromycin have antagonistic effects on each other when co-administered. These researches showed that following exposure to sub-inhibitory concentrations of clarithromycin or other antibiotics, an auto-regulatory transcriptional activator, WhiB7, was induced, resulting in resistance to clarithromycin, as well as cross-resistance to amikacin (Pryjma, et al., 2017). The *erm*(41) and *eis2* genes were identified as *whib7*-dependent genes which conferred resistance to clarithromycin and amikacin respectively (Pryjma et al., 2017).

The inducible *erm*(41) is a well-documented genetic determinant of macrolide resistance, especially clarithromycin (Nash, Brown-Elliott and Wallace, 2009). Although all three *M. abscessus* subspecies possess this inducible gene, the clarithromycin susceptibility pattern differs in them. This is because *M. abscessus* subsp. *massiliense* has a nonfunctional *erm*(41) gene, making it vulnerable to clarithromycin (Kim, et al., 2010b).

Acquired resistance in *M. abscessus* is often a result of spontaneous chromosomal mutation (Nessar, et al., 2012). A spontaneous single A1408G mutation in the 16S rRNA was associated with aminoglycoside resistance in *M. abscessus* (Prammananan et al., 1998), whereas mutations in either position 2058 or 2059 in the *rrl* gene are responsible for acquired macrolide resistance (Bastian, et al., 2011; Choi, et al., 2017).

2.11 Tetracycline

First discovered in the 1940s, tetracyclines inhibit the synthesis of protein by obstructing the aminoacyl-tRNA from binding to the ribosomal acceptor (A) site. As broad-spectrum antibiotics, tetracyclines are effective against a huge range of gram-positive and gram-negative bacteria (Chopra and Roberts, 2001).

Sadly, the emergence of tetracycline resistance followed the widespread use of these antibiotics in clinical practise as well as in animal feeds (Chopra and Roberts, 2001). Increased effort in research was then put into enhancing the understanding of the mechanisms of tetracycline resistance in the hope of salvaging the use of tetracyclines as a therapeutic agents. This resulted in the development of a new tetracycline derivatives, glycylcyclines (Chopra and Roberts, 2001).

2.12 Tigecycline

Tigecycline is the first glycylcycline with extended-spectrum properties to be developed to overcome the common mechanisms of antibiotic resistance (Peterson, 2008). Glycylcycline is a new class of antibiotics which harbours the central 4-ring carbocyclic skeleton. It was shown that the addition of a sidechain on the D ring increases its spectrum of activity and allows the evasion of tetracycline resistance mechanisms (George and Pankey, 2005). Following this discovery, tigecycline was developed by incorporating a 9-*t*-butyl-glycylamido

side chain at the 9th position of the D ring of minocycline (George and Pankey, 2005). This modification boosts the potency of tigecycline (Livermore, 2005).

Tigecycline functions by inhibiting protein synthesis in bacteria as it binds to the bacterial 30S ribosome and prohibits the integration of amino acid into the peptide chains (Greer, 2006; Zhanel, et al., 2012). Unlike tetracycline, this glycylcycline does not induce the expression of MabTetX, a drug modifying enzyme which is responsible for tetracycline resistance in *M. abscessus* (Rudra, et al., 2018). Tigecycline is also a poor substrate of this tetracycline-degrading enzyme (Rudra, et al., 2018).

As a broad-spectrum antibiotic, tigecycline is used in the treatment of patients with methicillin-resistant *Staphylococcus aureus*, extended-spectrum beta-lactamase producing bacteria and vancomycin-resistant enterococci infections (Noskin, 2005). Tigecycline has shown positive outcomes with RGM, particularly *M. abscessus* (Wallace, et al., 2002).

2.13 Tigecycline resistance

Over the years, the emergence of tigecycline resistant strains has threatened its efficacy, especially among Gram-negative bacteria (Sun, et al., 2013). Several mechanisms were associated with reduced susceptibility of tigecycline, such as the involvement of AcrAB efflux pumps (Sun, et al., 2013), mutation in *trm* encoding SAM-dependent methyltransferase (Chen, et al., 2014) and

modification of tigecycline by the TetX protein, a flavin-dependent monooxygenase (Moore, Hughes and Wright, 2005).

Unfortunately, the induction of *whiB7* (a transcriptional activator of natural antibiotic resistance) (Pryjma, et al., 2017) and RshA mutations (Ng, et al., 2018) had also been associated with the resistance to tigecycline in *M. abscessus*. Lee, et al. (2021) demonstrated that the *sigH* gene was linked to phenotypic resistance to tigecycline in *M. abscessus*. In line with the findings of Lee, et al. (2021), the *sigH* gene was shown to be overexpressed in *M. abscessus* upon exposure to tigecycline (Schildkraut, et al., 2022).

2.14 RshA and SigH

The regulation of gene expression following the signals from its host is essential for the mycobacteria to thrive under various environmental stresses such as heat and oxidative stress (Raman, et al., 2001). The transcription of the genes which are crucial in response to these stressors is mediated by SigH, an alternative sigma factor (Raman, et al., 2001). SigH expression in turn, is regulated by RshA, an anti-sigma factor (Song, et al., 2003).

In bacteria, alternative sigma factors play vital roles in controlling the expression of virulence genes which is crucial for a bacterial pathogen to cause an infection in the host as well as virulence-associated genes which enable the bacteria to survive in the harsh environment (Kazmierczak, et al., 2005).

Under unstressed conditions, the RshA binds to SigH and suppresses the SigH-dependent transcription (Song, et al., 2003). When the bacteria are subjected to stressful conditions such as redox stress, elevation in temperature or mutation in the conserved HXXXCXXC motif, the RshA-SigH interaction is disrupted, leading to the release of SigH and downstream phenotypic changes such as antibiotic resistance (Song, et al., 2003).

This interruption was demonstrated in the tigecycline-resistant mutant which harbours a C51R mutation that caused the first cysteine residue in the conserved HXXXCXXC motif to be changed to arginine (Ng, et al., 2018). A study by Ng, et al. (2020) described that the mutation in the conserved HXXXCXXC motif of RshA was also associated with the overexpression of the *sigH* gene which was verified by RT-qPCR analysis.

Using a tigecycline-resistant mutant (7C) derived from *M. abscessus* ATCC 19977, Ng, et al. (2018) linked a nonsynonymous C51R mutation in the *rshA* gene to tigecycline resistance. With further experimental evolution work, Lee, et al. (2021) identified two additional *rshA* mutations, namely K71E mutation in CL5A and an insertional mutation in CL6A, which were responsible for tigecycline resistance in the two mutants.

2.15 Efflux pumps

The efflux system was the last mechanism of resistance to be uncovered and they function by pumping solutes out of the cell. Efflux proteins derive their energy from ATP hydrolysis or proton motive force to pump the solutes out of the cell, against their concentration gradient. Efflux proteins can be present in both susceptible as well as resistant organisms. Typically, the overexpression of efflux pumps or increase in transport efficiency would lead to a resistance phenotype (Piddock, 2006).

While efflux proteins alone may not contribute to antibiotic resistance, Oethinger, et al. (2000) suggested that pumping harmful solutes to the external environment could effectively prolong the lifespan of the bacterium. This ability allows the mutations in other genes to accumulate, leading to a higher level of antibiotic resistance (Sun, Deng and Yan, 2014a).

First discovered in *Escherichia coli* in the 1980s, the Tet protein was documented to transport tetracycline out to the external environment, resulting in tetracycline being ineffective in Tet-positive *E. coli* strains (McMurry, Petrucci and Levy, 1980). Since then, there was increasing interest on the role of efflux pumps in antibiotic resistance. Five families of efflux proteins which were categorised based on the similarity of their sequences, energy source, number of parts and transmembrane regions, were identified (Table 2.3).

Table 2.3: The five families of efflux pumps

Family of efflux pump	Reference
Adenosine triphosphate (ATP)-binding cassette (ABC) superfamily	(Lubelski, Konings and Driessen, 2007)
Resistance-nodulation-division (RND) family	(Tseng, et al., 1999)
Small multidrug resistance (SMR) family	(Yong and Saier, 2001)
Major facilitator superfamily (MFS)	(Law, Maloney and Wang, 2008)
Multidrug and toxic compound extrusion (MATE) family	(Kuroda and Tsuchiya, 2009)

2.16 Efflux pumps in *M. abscessus*

In mycobacteria, efflux pumps are known to work in synergy with other intrinsic resistance mechanisms such as the low penetrance of the mycobacterial cell wall (Jarlier and Nikaido, 1994) and the production of drug modifying enzymes. The swelling interest to investigate the role of efflux pumps in multidrug resistance in mycobacterium is evidenced by the multiple studies done. It was reported that *Mycolicibacterium fortuitum*, an opportunistic human pathogen, possesses a Tap efflux pump, responsible for tetracycline resistance (Aínsa, et al., 1998). The first efflux pump discovered in *Mycobacterium smegmatis* is LfRA whose overexpression leads to ciprofloxacin resistance (Takiff, et al., 1996). A study by Pasca, et al. (2005) demonstrated that the *mmpL7* gene of *Mycobacterium tuberculosis* caused isoniazid efflux when overexpressed in *Mycobacterium smegmatis*.

Although the studies on efflux pumps in *M. abscessus* are limited, a handful of studies such as by Guo, et al. (2020) and Vianna, et al. (2019) documented the involvement of efflux pumps (*MAB_2355c*, *MAB_1409c*, *MAB_1846* and *MAB_3142* and *MAB_1409* respectively) in clarithromycin resistance in *M. abscessus*. *MAB_3142* and *MAB_1409* belong to the Major Facilitator Super Family (Rindi, 2020). Whereas *MAB_2355c* and *MAB_1846* are putative ABC transporter ATP-binding protein and *MAB_1409c* is annotated as a putative drug antiporter protein precursor.

CHAPTER 3

METHODOLOGY

The overall design of the experiment is described in Figure 3.1

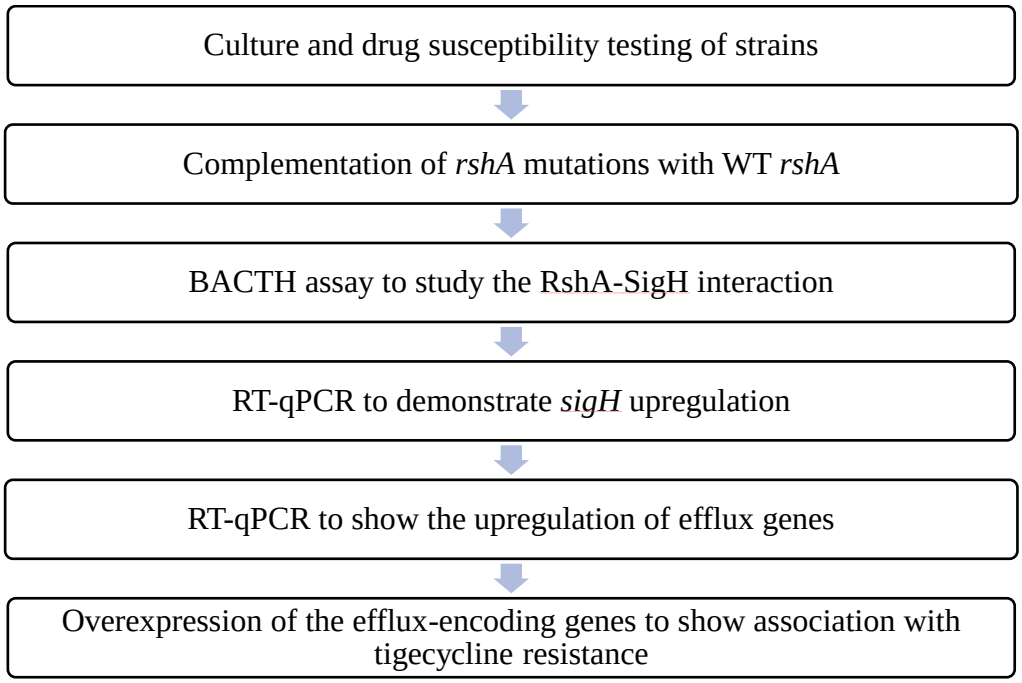


Figure 3.1: Overall design of the experiment of the research project. wt: wild type; BACTH: bacterial adenylate cyclase two hybrid

This study was carried out to investigate the relationship between *rshA* mutations and efflux pumps with tigecycline resistance in *M. abscessus*.

3.1 Culture and storage of bacterial strains

Table 3.1: The *M. abscessus* strains used in this study

Strain	Plasmid	Phenotype
ATCC 19977	-	Tigecycline-sensitive
CL5A	-	Tigecycline-resistant
CL5A-3542c wt	pMV261-*MAB_3542c*^{wt}	Tigecycline-sensitive
CL5A-pMV261	pMV261	Tigecycline-resistant
CL6A	-	Tigecycline-resistant
CL6A-3542c wt	pMV261-*MAB_3542c*^{wt}	Tigecycline-sensitive
CL6A-pMV261	pMV261	Tigecycline-resistant
ATCC 19977-1299c wt	pMV261-*MAB_1299c*^{wt}	Tigecycline-sensitive
ATCC 19977-1395 wt	pMV261-*MAB_1395*^{wt}	Tigecycline-sensitive
ATCC 19977-1396 wt	pMV261-*MAB_1396*^{wt}	Tigecycline-sensitive

The tigecycline-resistant mutants (CL5A and CL6A) selected from the tigecycline-susceptible ATCC 19977 (American Type Culture Collection) were gifts from Ng Hien Fuh and Lee Col Lin of FMHS, UTAR. All two mutants possessed the *rshA* mutations at different loci which conferred tigecycline resistance (Table 4.1).

All the strains used in this study were cultured routinely at 30 ℃ to 35 ℃ on nutrient agar or broth (Becton Dickinson [BD]). The culture media which were used in the experiments in this study were prepared as described in Appendix A. The bacterial strains were frozen in broth with 15% glycerol (Sigma-Aldrich) at -80 ℃ until required for use.

3.2 Antimicrobial susceptibility testing

The wt ATCC 19977 and mutants transformed with empty plasmids were included as controls in this study. Stokes disk diffusion and Etest were performed in this study for antimicrobial susceptibility testing.

3.2.1 Stokes disk diffusion

In Stokes disc diffusion, the test and control strains were adjusted to 0.5 McFarland density and evenly streaked onto the two halves of a pre-poured cation-adjusted Mueller-Hinton Agar (CAMHA) plate with a gap no more than 5 mm between the two bacterial lawns, in which, a tigecycline 15 µg disc (BD) was placed at the centre of the gap. The zones of inhibition were measured for each plate after being incubated at 30 ℃ for 3-4 days.

3.2.2 Etest

For the Etest, the test and control strains were adjusted to 2 McFarland density to make the bacterial lawn on two separate CAMHA plates. An Etest tigecycline strip (Biomerieux) was placed at the centre of each bacterial lawn on the CAMHA plates. The plates were incubated at 30 ℃ for 3-4 days. The MIC was read as the lowest concentration of antibiotic that inhibits the visible growth of the bacterium.

3.3 Molecular biology analyses

3.3.1 Preparation of nucleic acid

The total DNA was extracted from the plate cultures with the ZR Fungal/Bacterial DNA Miniprep (Zymo Research) according to the instructions from the manufacturer.

Log phase broth cultures in CAMHB were prepared in triplicates and incubated with shaking at 37 ℃, at an optical density of 600 nm [OD_{600}] of 0.1 to 1. The RNA was prepared using the RNeasy Mini Kit (Qiagen) following the manufacturer's protocol. After the bacterial cells in the ZR BashingBead Lysis tubes (Zymo Research) were lysed mechanically with the RLT buffer through high-speed shaking using the Vortex Genie 2 (Scientific Industries). The concentration of the RNA extracted was measured with NanoDrop One.

After that, the extracted RNA was treated with DNase digestion in solution followed by Ethylenediaminetetraacetic acid and heat inactivation at 75 ℃ for 10 minutes.

3.3.2 End point PCR

For all the end-point PCR analyses carried out in this study, the following conditions were practiced. The PCR cocktail which included 1×GoTaq Green Mastermix (Promega), 0.2 µM of both forward and reverse primers (Table 3.2),

nuclease-free water and 10 ng of purified DNA, was prepared. Amplification was performed using this profile: 1 cycle of 95 ℃ for 10 minutes, 35 cycles of 95 ℃ for 30 seconds, annealing temperature described in Table 3.2 for 30 seconds, 72 ℃ for 60 seconds and finally, 1 cycle of 72 ℃ for 10 minutes, using the Veriti Thermal Cycler (Applied Biosystems). In each run, a no-template control was added in, and the PCR products were analysed using gel electrophoresis with 1.5 % agarose gel. The agarose gel stained with SYBR Safe (Thermo Scientific) was visualised under the ultraviolet (UV) using the Biospectrum 410 Gel Documentation System (UVP). The PCR products were to be sent for Sanger Sequencing (Apical Scientific Ltd) if necessary.

3.3.3 RT-qPCR

Reverse-transcription to cDNA was performed using the ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo). The qPCR cocktail containing 1×Thunderbird SYBR qPCR Mix (Toyobo), 0.2 µM of both forward and reverse primers (Table 3.2), nuclease-free water and 2 µl of undiluted cDNA, was prepared and the amplification was carried out with the following thermal cycling profile: 1 cycle of 95 ℃ for 60 seconds, followed by 40 cycles of 95 ℃ for 15 seconds and 60 ℃ for 60 seconds, in a Rotor-Gene Q Real-time PCR Cycler (Qiagen). Triplicate samples were tested in technical duplicates for CL5A and CL6A. The expression of the *sigH* gene was quantified in relative to *MAB_1512* following the mathematical model described by (Pfaffl, 2001), as it was identified as the most stably expressed gene by RefFinder among the other two candidates (*MAB_4545* and *MAB_4107c*).

In all the RT-qPCR experiments, no-reverse transcriptase and no-template controls were included. Standard curves for *MAB_3543c* and the housekeeping genes were generated to ascertain the efficiency of the amplification using serially diluted cDNA (the efficiencies of the described genes ranged from 0.9-1.1). Analyses of the melt curve and gel electrophoresis were used to evaluate the amplification specificity of the primers which were designed using Primer-BLAST (Table 3.2).

3.3.4 Bacterial Adenylate Cyclase Two Hybrid (BACTH) assay

The RshAmut (RshA from tigecycline-resistant mutant)-SigH interaction in *M. abscessus* was studied using the Bacterial Adenylate Cyclase Two-Hybrid System (BACTH) (Euromedex). In essence, the BACTH kit provided one plasmid carrying the T25 subunit (pKT25) and another carrying the T18 subunit (pUT18) of the adenylate cyclase. In-frame cloning of the *rshA* gene (amplified from the wt ATCC 19977 or CL5A) into the pUT18 plasmid and the *sigH* gene (amplified from the wt ATCC 19977) into the pKT25 plasmid was carried out. The presence of an interaction between the target proteins would restore the activity of the adenylate cyclase in the reporter *Escherichia coli* BTH101 strain and hence, cAMP would be synthesised and bind to CAP. The formation of cAMP/CAP complex would allow the genes which are involved in lactose catabolism to be expressed and blue colonies would be seen on the LB agar supplemented with X-GAL, IPTG and the appropriate antibiotics (ampicillin and kanamycin).

3.3.4.1 Cloning of target genes

The target gene from the tigecycline-resistant mutant, CL5A was PCR-amplified in the Veriti Thermal Cycler with the mycobacterial colony PCR profile (Table 3.2) with the annealing temperature (Table 3.2). Following purification of the amplicons with the QIAquick PCR Purification Kit (Qiagen), the amplicons and the respective plasmids were double digested with the suitable restrictive enzymes described in Table 3.2. The digested products were purified with the QIAquick PCR Purification Kit and then subjected to ligation with the presence of T4 DNA ligase (NEB).

The wt *sigH* was cloned into plasmid pKT25 (pKT25-*MAB_3543c*^{wt}) which expresses the kanamycin resistance selectable marker and RshAmut from CL5a was cloned into plasmid pUT18 (pUT18-*MAB_3542c*^{CL5Amut}) which expresses the ampicillin resistance selectable marker. The recombinant plasmids were then transformed into XL10-Gold ultracompetent cells. The positive colonies were selected on LB agar supplemented with 30 mg/L kanamycin (Gold Biotechnology) (for transformants with the pKT25-*MAB_3543c*^{wt}) and 100 mg/L ampicillin (Gold Biotechnology) (for transformants with the pUT18-*MAB_3542c*^{CL5Amut}). The colonies were screened with colony PCR with the annealing temperature (Table 3.2) and primers (Table 3.2).

3.3.4.2 Co-transformation of recombinant plasmids into the reporter strain, an adenylate cyclase deficient (*cya*) *E. coli* mutant, BTH101

After verification with Sanger sequencing, the recombinant plasmids of interest (pKT25-*MAB_3543c*^{wt} and pUT18-*MAB_3542c*^{CL5Amut}) were co-transformed into the reporter strain, an adenylate cyclase deficient (*cya*) *E. coli* mutant, BTH101, through heat shock at 42 ℃ for 30 seconds and plated onto LB agar supplemented with 50 mg/L kanamycin, 100 mg/L ampicillin, 200 mg/L X-Gal and 1mM of IPTG. The LB/X-Gal/IPTG plates were incubated at 37 ℃ for 16 hours. After 16 hours, cell seeding was done in LB/X-Gal/IPTG.

3.3.4.3 Screening

On top of a positive control (BTH101 co-transformed with SigH-RshA wt), both positive and negative controls which came as part of the kit were also included. The development of the blue colour of the reporter BTH101 strain was observed and compared with the three other control strains every two hours.

Table 3.2: Primers used in this study

Primers	Sequence (5'-3')	Target	Estimated size (bp)	Annealing temperature (˚C)	Purpose	Reference
1395-F	CTCGTCATTGGTATTGCGCC	*MAB_1395*		60	RT-qPCR	This study
1395-R	GAACCACGAACACCTTGTCG					
1396-F	TGGCCTAGTGGTTTTGGGTG	*MAB_1396*		60	RT-qPCR	This study
1396-R	AGAACATCATGGGCAGACCG					
1299c-F	CTGCACACTTCATCGTTGCC	*MAB_1299c*		60	RT-qPCR	This study
1299c-R	ACGAAGTAGCTGGAGGTCAC					
1395-F	TAAG**GAATT[1]C**GTGCGTGCGCTG AGCA	*MAB_1395*	1296	72	Cloning	This study
1395-R	CCGTAAGCTT[1]TCAGGCAGCGACA CGATTTC					
1396-F	TAAG**GGATCC[2]**aGTGCGACCTGAA TATCCG	*MAB_1396*	1486	72	Cloning	This study
1396-R	CCGTAAGCTT[2]CTAATCTTCGACG GAGACC					
1299c-F	TAAG**CTGCAG[3]**TGATTGCCCGTCT GCG	*MAB_1299c*	1220	72	Cloning	This study
1299c-R	CCGTTTCGAA[3]TTAGCCGGTGGAC ACCG					

Table 3.2 (continued): Primers used in this study

Primers	Sequence (5'-3')	Target	Estimated size (bp)	Annealing temperature (˚C)	Purpose	Reference
B2H-3543c-F	AT**TCTAGA**[4]GGCGTGCCTCGAACGTCC	*MAB_3543c*	721	62	Cloning	This study
B2H-3543c-R	ATGGTACC[4]CGTGCCCGGCCCTCCGC					
B2H-3542c-F	AT**TCTAGA**[5]GACCGACGGTGAACTCAAGAAGA	*MAB_3542c*	527	62	Cloning	This study
B2H-3542c-R	ATGGTACC[5]CGGGAGTTCTCGGCCCGCTG					
1395TF-F	CTGCTCATCCTCGCCGTG	*MAB_1395*	1477	60	Verification of whole-genome sequencing findings	This study
1396TF-F	CCGCTGGCCTAGTGGTTTT	*MAB_1396*	1666	60	Verification of whole-genome sequencing findings	This study

Table 3.2 (continued): Primers used in this study

Primers	Sequence (5'-3')	Target	Estimated size (bp)	Annealing temperature (˚C)	Purpose	Reference
1299TF-F	GCCGCACTGGGATCCTTC	*MAB_1299c*	1402	60	Verification of whole-genome sequencing findings	This study
3542screen-F 3542screen-R	CTGTTATGTCGCGGTTGCAC ACCGGCATGAGAGACTGGAT	*MAB_3542c*	596	60	Screening for *MAB_3542c* mutations in the mutants	(Ng, et al., 2018)
pMV261-F pMV261-R	CCAGCGTAAGTAGCGGGGTT AGTCTTTCGACTGAGCCTTTCG	Partial pMV261	194	60	Screening for the pMV261 plasmid	(Ng, et al., 2018)
1512-F 1512-R	GGAGGTTGTCGAGGTTCAGG GATCGCGGGTGAATTTGTCG	Partial *MAB_1512*	194	60	RT-qPCR	(Ng, et al., 2018)
4545-F 4545-R	AAGGTGCTACGTGCCATGAA TCATAGCGGATCACGACGAC	Partial *MAB_4545c*	218	60	RT-qPCR	(Ng, et al., 2018)
4107-F 4107-R	TTAGTGATGAGTGCTGGGCG TGGGACGCATCGGAATATGG	Partial *MAB_4107c*	162	60	RT-qPCR	(Ng, et al., 2018)

The sequence of the forward restriction enzyme recognition site is in bold while the sequence of the reverse restriction enzyme recognition site is underlined.

[1]The forward restriction enzyme used for *MAB_1395* was *Eco*RI and the reverse restriction enzyme was *Hin*dIII

[2]The forward restriction enzyme used for *MAB_1396* was *Bam*HI and the reverse restriction enzyme was *Hin*dIII

[3]The forward restriction enzyme used for *MAB_1299c* was *Pst*I and the reverse restriction enzyme was *Bst*BI

[4]The forward restriction enzyme used for *MAB_3543c* was *Xba*I and the reverse restriction enzyme was *Kpn*I

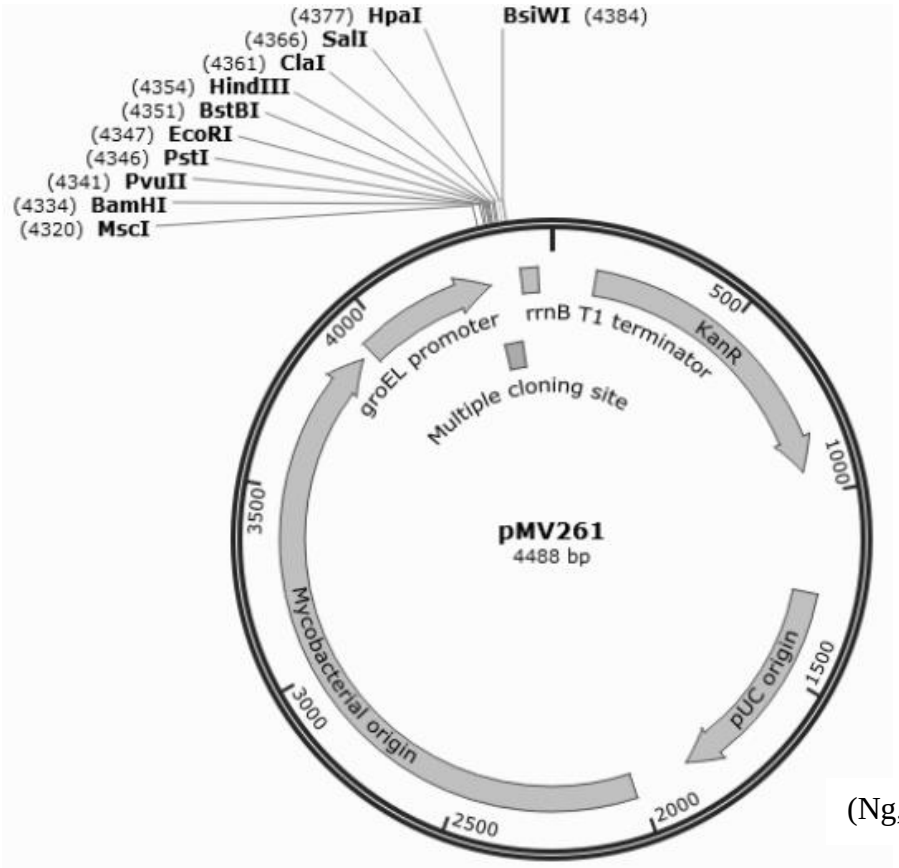
[5]The forward restriction enzyme used for *MAB_3542c* was *Xba*I and the reverse restriction enzyme was *Kpn*I

Table 3.3: Plasmids used in this study

Plasmid	Description
pMV261 (Figure 3.2)	A *Mycobacterium-Escherichia coli* shuttle vector with the mycobacterial origin of replication and pUC origin. Upstream to the constitutive *groEL* promoter, the target genes would be individually cloned into the multiple cloning site. Contains the *kanR* gene which leads to kanamycin resistance in the bacterial hosts (Stover, et al., 1991)
pMV261-*MAB_3542c*^{wt}	pMV261 carrying the wt *rshA* gene
pUT18	A derivative of the high copy number vector pUC19 which expresses an ampicillin resistance selectable marker. Encodes the T18 fragment (amino acids 225 to 399 of CyaA). Expresses chimeric proteins for heterologous polypeptide to fuse to the N-terminal end of T18 (*BACTH System Kit Bacterial Adenylate Cyclase Two-Hybrid System Kit*)
pUT18-*MAB_3542c*^{CL5Amut}	pUT18 carrying the *rshA* gene from CL5A
pUT18-*MAB_3542c*^{wt}	pUT18 carrying the *rshA* gene from the wt strain
pKT25	A derivative of the low copy number vector pSU40 which expresses a kanamycin resistance selectable marker. Encodes the T25 fragment (first 224 amino acids of CyaA). Contains a multicloning site sequence for the construction of in-frame fusions at the C-terminal end of the T25 polypeptide
pKT25-*MAB_3543c*^{wt}	pKT25 carrying the *sigH* gene from the wt strain
pMV261-*MAB_1299c*^{wt}	pMV261 carrying the efflux gene *MAB_1299c*

Table 3.3 (continued): Plasmids used in this study

Plasmid	Description
pMV261-*MAB_1395*^{wt}	pMV261 carrying the efflux gene *MAB_1395*
pMV261-*MAB_1396*^{wt}	pMV261 carrying the efflux gene *MAB_1396*



(Ng, 2019)

Figure 3.2: The graphical representation of the plasmid, pMV261. This is a *Mycobacterium-Escherichia coli* shuttle vector. The target efflux genes were individually cloned into the multiple cloning site, using the promoter, *groEL*. pMV261 also expresses a kanamycin resistance selectable marker.

3.4 Cloning and transformation in efflux gene dosage studies

3.4.1 Molecular cloning of target genes

The target efflux-encoding genes were PCR-amplified in the Veriti Thermal Cycler from the wt ATCC 19977 DNA with the Q5 High-Fidelity 2×Master Mix (NEB) and primers described in Table 3.2. The following profile was used: 1 cycle of 98 ℃ for 30 seconds, 35 cycles of 98 ℃ for 10 seconds, annealing temperature described in Table 3.2 (72 ℃) for 30 seconds and 72 ℃ for 30 seconds, and 1 cycle of 72 ℃ for 2 minutes. Cleanup of the amplicons was performed using QIAquick PCR Purification Kit (Qiagen). Subsequently, double digestion was performed using the suitable restriction enzymes (Table 3.2) and CutSmart buffer (NEB). The purified fragments which were purified with the QIAquick PCR Purification Kit were then ligated at 5:1 insert:vector molar ratio with the T4 DNA ligase (NEB). Five µl of the ligated products were transformed into the NEB 5-alpha competent *E. coli* cells through heat shock for 30 seconds at 42 ℃. The transformed cells were plated onto Luria-Bertani (LB) agar (First Base) supplemented with 30 mg/L kanamycin (Gold Biotechnology) and incubated overnight at 37 ℃. The positive colonies were selected after screening with colony PCR (see section 3.3.2) and the recombinant plasmids were propagated and purified from the LB broth (First Base) cultures which were supplemented with 30 mg/L of kanamycin with the DNA-spin Plasmid Purification Kit (iNtRON Biotechnology). Sanger sequencing confirmed that no mutations were introduced.

3.4.2 Transformation of the recombinant plasmids into *M. abscessus*

3.4.2.1 Preparation of electro-competent *M. abscessus* cells

The electro-competent *M. abscessus* cells were prepared as described previously (Broussard, 2009). In short, after the *M. abscessus* cells were harvested at log phase (OD_{600} of 0.1-1) and washed for three times and resuspended with cold sterile 10% (v/v) glycerol, the electro-competent cells were frozen on a cold block and stored in -80 ℃ in aliquots of 100 μl.

3.4.2.2 Electroporation of the recombinant plasmids into the electro-competent *M. abscessus* cells

In a 0.2 cm electroporation cuvette (Bio-Rad), the recombinant plasmids were electroporated into the electro-competent *M. abscessus* cells using Eporator (Eppendorf) at 2500 V. The cells were then plated onto the Middlebrook 7H10 agar (BD) supplemented with 50 mg/L kanamycin. The plates were incubated for 3-5 days following recovery in the Middlebrook 7H9 broth (BD). The plasmid-positive clones were screened using colony PCR.

3.5 BLAST analyses

The corresponding orthologs of the efflux genes in *M. tuberculosis*, *M. smegmatis* and other bacteria were identified using BLASTp (protein Basic Local Alignment Search Tool).

3.6 Statistical analysis

Quantitative experiments were all carried out in triplicates and the data obtained was recorded in mean ± one standard deviation. The difference in mean between two groups was analysed using the two-sample t-test and p-value < 0.05 as the cut off value for statistical significance, with the GraphPad Prism 5 software**.**

CHAPTER 4

RESULTS

4.1 Characterisation of mutants

Two tigecycline-resistant mutants (CL5A and CL6A) with *rshA* mutations were used in this study. Both strains were derived from the tigecycline-sensitive parental ATCC 19977 strain. The resistance phenotype of the mutants was demonstrated and confirmed by significantly smaller growth inhibition zone sizes in comparison to the wt ATCC 19977 in Stokes disk diffusion test. Using Etest, the MIC of the mutants were eight-fold higher (tigecycline MIC: 2 mg/L) than the MIC of the wt ATCC 19977 strain (tigecycline MIC: 0.25 mg/L). According to EUCAST: AST of Bacteria (2022) using the non-species related breakpoints, the mutants were confirmed to be resistant against tigecycline (sensitive ≤0.5mg/L, resistant >0.5mg/L).

4.2 Complementation of CL5A and CL6A with wt *rshA*

CL5A and CL6A possessed *rshA* mutations in different loci (Table 4.1) (Figure 4.1) which conferred tigecycline resistance in the mutants.

In this study, CL5A and CL6A were complemented with the wt *rshA* to demonstrate the causal relationship between *rshA* mutations and the phenotypic resistance to tigecycline in *M. abscessus*. The PCR products of the wt *rshA* gene

(*MAB_3542c*) were cloned into a *Mycobacterium-E. coli* shuttle vector, pMV261 which contains KanR, and the generated recombinant plasmids pMV261-*MAB_3542c*^{wt}. pMV261-*MAB_3542c*^{wt} were transformed into the electrocompetent CL5A and CL6A competent cells, producing CL5A-3542c wt and CL6A-3542c wt. Screened and selected using colony PCR, the PCR products were sent for Sanger sequencing to verify that the mutants were complemented with wt *rshA*. Mutants carrying the empty plasmids (CL5A-pMV261 and CL6A-pMV261) were included as controls.

As expected, complementation of the tigecycline-resistant mutants with the wt *rshA* successfully restored their tigecycline susceptibility. Using Stokes agar plate disk diffusion, the tigecycline inhibition zone sizes of the strains complemented with the wt *rshA* were remarkably bigger as compared to the mutants with the empty plasmid (Figure 4.2).

This was subsequently confirmed using Etest in which the MIC of the complemented strains was decreased to 0.25 mg/L while the mutants with the empty plasmid retained the MIC of 2 mg/L (Table 4.2).

These results demonstrated that the *rshA* mutations possessed by CL5A and CL6A were responsible for the tigecycline resistant phenotype seen in the mutants.

Table 4.1: *rshA* mutations

Strain	DNA mutation	MIC (mg/L)
CL5A	a211g	2
CL6A	13_14 ins aactc	2

The *rshA* mutations in the respective tigecycline-resistant mutants.

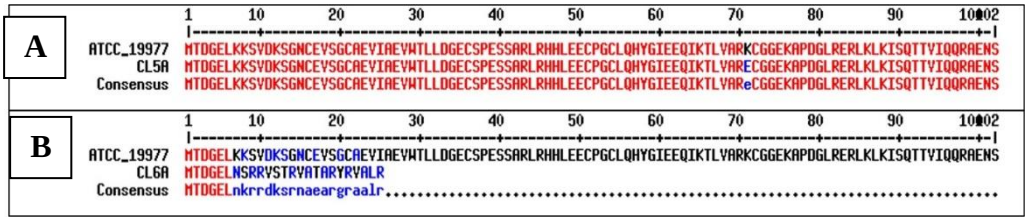


Figure 4.1: The tigecycline-resistant mutants were aligned with ATCC 19977 parental strain to show the *rshA* mutations. Red letters indicate identical amino acids between the parental ATCC 19977 strain and the mutants. Black and blue letters indicate dissimilar amino acids.

Table 4.2: The tigecycline zone diameters and MIC of complemented strains and mutants

Strain	Tigecycline Zone Diameter (mm)	MIC (mg/L)
CL5A-3542c wt	39	0.25
CL5A-pMV261	21	2
CL6A-3542c wt	41	0.25
CL6A-pMV261	22	2

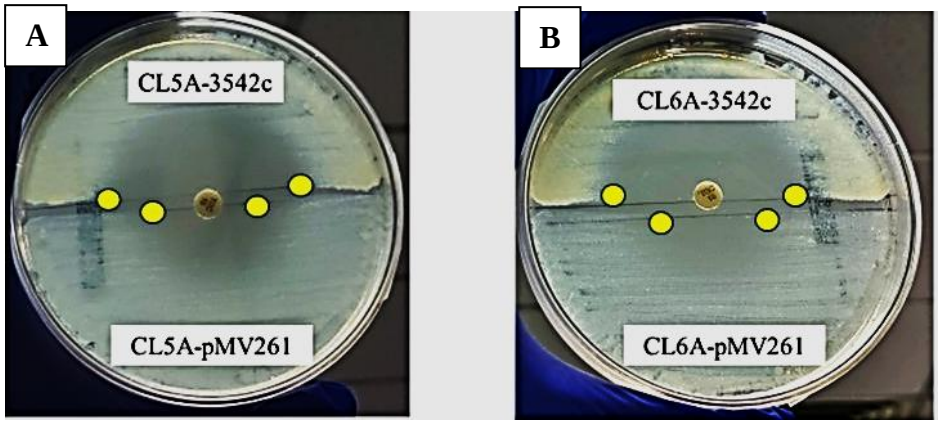


Figure 4.2: Antimicrobial susceptibility testing of CL5A and CL6A complemented with wt *rshA* using Stokes agar plate disk diffusion. The yellow dots represent the inhibition zone size. (A) Tigecycline zone sizes of CL5A-3542c wt (top) vs CL5A-pMV261 (bottom); (B) Tigecycline zone sizes of CL6A-3542c wt (top) vs CL6A-pMV261 (bottom)

4.3 BACTH assay to demonstrate the disruption of RshA-SigH interaction

The BACTH assay which was designed to study protein-protein interaction was utilised in this study to investigate the effect of the *rshA* mutation in CL5A on the interaction between the RshA and SigH factors.

CL6A was not subjected to BACTH analysis as the insertional mutation in CL6A caused a frameshift mutation which abolished the HXXXCXXC motif. Hence, it is reasonable to assume that the truncation of the RshA protein would disrupt its interaction with SigH.

Interestingly, the development of the blue colour in the *E. coli* reporter strain BTH101 which was co-transformed with pKT25-*MAB_3543c*^{wt} and pUT18-*MAB_3542c*^{CL5Amut} was visibly slower than the positive control provided by the kit as well as the BTH101 reporter strain transformed with the wt *rshA* (Figure 4.3). This finding demonstrated that the interaction between the CL5A RshA and SigH was indeed retarded as compared to the control (*sigH* and the wt *rshA*) and thus confirming the hypothesis that *rshA* mutation in CL5A disrupted its interaction with SigH.

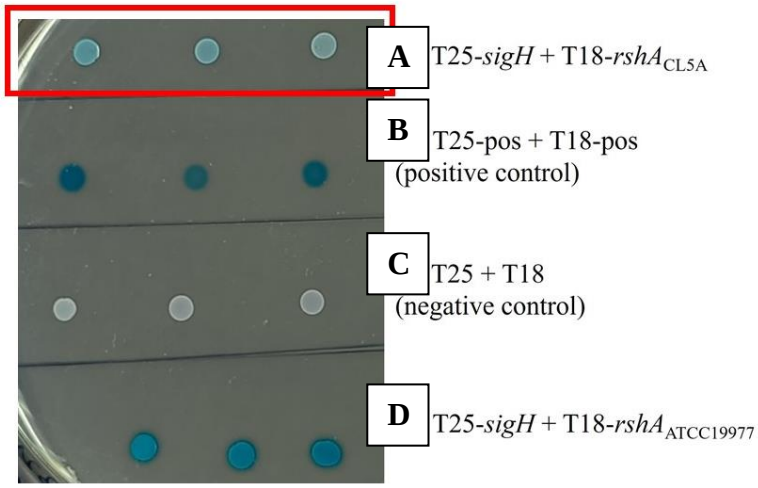


Figure 4.3: Difference in the development of blue colour in the *E. coli* reporter strain BTH101 transformed with the *rshA* from CL5A, positive and negative controls and the *E. coli* reporter strain BTH101 transformed with the wt *rshA*. (A) The red rectangle showed that *E. coli* reporter strain BTH101 co-transformed with the *rshA* from CL5A (T18-rshA$_{CL5A}$), and wt *sigH* (T25-sigH) (B) Positive control provided by the kit (T25-pos and T18-pos) (C) Negative control provided by the kit (T25 and T18) (D) *E. coli* reporter strain BTH101 transformed with wt *rshA* (T18-rshA$_{ATCC19977}$) and wt *sigH* (T25-sigH).

4.4 RT-qPCR to demonstrate *sigH* upregulation

Following the demonstration of the retarded interaction between the RshA and SigH protein in CL5A, it was hypothesised that the *sigH* gene would be upregulated in the mutants with *rshA* mutations due to the decreased inhibition SigH following the disruption of RshA-SigH interaction. Hence, RT-qPCR was used to quantify the mRNA levels of *sigH* gene in CL5A and CL6A.

The results from the RT-qPCR analysis of the *sigH* gene showed that it was significantly upregulated in (a) CL5A (p-value < 0.05, fold-change = 5.99) and (b) CL6A (p-value < 0.05, fold-change = 9.33) as compared to the expression of the *sigH* gene in ATCC 19977 (Table 4.3) (Appendix B and C).

Table 4.3: RT-qPCR analysis of *sigH* gene

Strain	*sigH* (fold-change; p-value)
CL5A	5.99; < 0.05
CL6A	9.33; < 0.05

4.5 Efflux-encoding genes in CL5A and CL6A

Three genes (*MAB_1299c*, *MAB_1395* and *MAB_1396*) annotated to be putative drug/multidrug transporters were discovered to be upregulated in a previously isolated tigecycline-resistant mutant 7C (Ng, et al., 2020). This suggests that efflux genes may be involved in the mechanism of tigecycline resistance in *M. abscessus* alongside the *rshA* mutation in 7C. Thus, RT-qPCR was performed to quantify the mRNA level of these three efflux genes in CL5A and CL6A, which also possessed *rshA* mutations and displayed tigecycline-resistance phenotype.

The purpose of this experiment was to determine if the efflux pumps were also upregulated in the mutants with *rshA* mutations.

The results showed that these three efflux-encoding genes were all significantly upregulated in CL5A (a) *MAB_1299c* (p-value < 0.01, fold-change = 158.6) (b) *MAB_1395* (p-value < 0.01, fold-change = 53.7) (c) *MAB_1396* (p-value < 0.01, fold-change = 133.3). Whereas for CL6A, (a) *MAB_1299c* (p-value < 0.05, fold-change = 12.6) was significantly upregulated but for (b) *MAB_1395* (p-value = 0.057, fold-change = 4.8) and (c) *MAB_1396* (p-value = 0.06, fold-change = 7.9), the upregulations were borderline significant (Table 4.4) (Appendix D-G). The RT-qPCR results suggested that efflux genes could have played a role in the tigecycline resistance seen in the mutants CL5A and CL6A.

Table 4.4: RT-qPCR analysis of the efflux-encoding genes

Strain	***MAB_1299c* (fold-change; p-value)**	***MAB_1395* (fold-change; p-value)**	***MAB_1396* (fold-change; p-value)**
CL5A	158.6; < 0.01	53.7; < 0.01	133.3: < 0.01
CL6A	12.6; < 0.05	4.8; 0.057	7.9; 0.06

4.6 Overexpression of efflux-encoding genes in wt ATCC 19977

The demonstration of upregulation of efflux genes, namely *MAB_1299c*, *MAB_1395* and *MAB_1396*, in CL5A and CL6A suggested that efflux mechanisms could contribute to the phenotypic resistance to tigecycline in *M. abscessus*. Therefore, these three efflux-encoding genes were overexpressed in the wt ATCC 19977 to see whether the overexpression of these genes would lead

to resistance to tigecycline in the tigecycline susceptible *M. abscessus* ATCC 19977.

Using the mycobacterial origin of replication, the plasmids carrying the efflux genes (pMV261-*MAB_1299c*^{wt}, pMV261-*MAB_1395*^{wt} and pMV261-*MAB_1396*^{wt}) were multiplied in the mycobacterial host *M. abscessus* ATCC 19977 (Figure 3.2). This increased the copy number of the target efflux genes causing them to be overexpressed with transcription from the promoter, *groEL*. The parental ATCC 19977 strain transformed with the empty pMV261 was included as control in this experiment.

Disappointingly, the overexpression of the three efflux encoding genes in ATCC 19977 did not show an increase in tigecycline resistance as demonstrated by the zones of inhibition in Stokes disk diffusion test (Table 4.5).

Table 4.5: Tigecycline susceptibility patterns of ATCC 19977 transformed with recombinant plasmids carrying efflux genes *MAB_1299c*, *MAB_1395* and *MAB_1396* (disk diffusion)

Strain	Plasmid	Tigecycline zone diameter (mm)	
		Target strain	Control (strain carrying empty plasmid pMV261)
ATCC 19977-1299c wt	pMV261-*MAB_1299c*^{wt}	34	34
ATCC 19977-1395 wt	pMV261-*MAB_1395*^{wt}	32	32
ATCC 19977-1396 wt	pMV261-*MAB_1396*^{wt}	33	33

CHAPTER 5

DISCUSSION

The upward trend of pulmonary infections caused by NTM is increasingly worrying. Among the NTM, *M. abscessus* is one of the most clinically significant and commonly identified mycobacteria which is responsible for chronic pulmonary infections suffered by patients, often with underlying chronic pulmonary diseases such as cystic fibrosis (Degiacomi, et al., 2019).

M. abscessus is notoriously associated with intrinsic and acquired resistance to most commonly available antibiotics, making treatment a challenge (Victoria, et al., 2021). Fortunately, tigecycline, a vital rescue antibiotic for several bacterial infections has demonstrated good activities *in vitro* and *in vivo*. Presently, it is regarded as one of the most effective treatment options for patients with *M. abscessus* infections (Wallace, et al., 2014; Ferro, et al., 2016). Tigecycline is also recently included in the initial phase of *M. abscessus* treatment alongside intravenous amikacin, imipenem, and oral clarithromycin, as recommended by the British Thoracic Society (Victoria, et al., 2021).

However, like many other fundamental antibiotics, the emergence of clinical *M. abscessus* strains with the tigecycline-resistant phenotype has been documented (Ananta, et al., 2018), threatening the efficacious lifespan of this antibiotic. The mechanisms of tigecycline resistance in *M. abcessus* are not well understood and only a handful of studies had explored the possible causes which could explain

the tigecycline resistance phenotype in this multidrug resistant mycobacterium. If left unattended, undesirable consequences may ensue.

In this study, *rshA* mutations and efflux pumps were further explored to have a better understanding on their roles in tigecycline resistance in *M. abscessus*.

5.1 Causal relationship of the *rshA* mutations and tigecycline resistance in *M. abscessus* established using gene complementation

A previous study had identified RshA mutations to be tigecycline resistance determinants in *M. abscessus* through the characterisation of a tigecycline-resistant mutant, 7C (Ng, et al., 2018). Whereas, in another study, CL5A and CL6A were identified to possess RshA mutations at different loci (Lee, et al., 2021). All three strains were isolated using experimental evolution and derived from the tigecycline sensitive parental ATCC 19977 strain. 7C possessed a C51R mutation, whereas CL5A harboured a K71E mutation and CL6A had a frameshift mutation which introduced a stop codon prematurely, resulting in a truncated gene

While the role of RshA as an anti-sigma factor may be well studied in other mycobacteria such as *M. tuberculosis*, studies on this genetic tigecycline resistance determinant in *M. abscessus* is lacking. Hence, in this study, the tigecycline-resistant mutants CL5A and CL6A, were further characterised to gain deeper insight on the role of RshA mutations in tigecycline resistance in *M. abscessus*.

Complementation of CL5A and CL6A with the pMV261 plasmid carrying the wt *rshA* gene effectively restored the tigecycline sensitivity phenotype (Figure 4.2) in the mutants. The results implied that the *rshA* mutations were responsible for the tigecycline resistance phenotypes in both CL5A and CL6A.

As *M. abscessus* possesses only a single copy of the *rshA* gene (Ng, et al., 2020), this could explain why a single-step mutation in the *rshA* gene could cause the MIC of tigecycline in the mutants to increase by eight-fold, from 0.25 mg/L to 2 mg/L. The outcome of this study was in good agreement with the previous work by Ng, et al. (2018) which demonstrated the T151C → C51R mutation in the *rshA* gene as a possible tigecycline resistance determinant in *M. abscessus*.

The anti-sigma factor, RshA which supresses SigH, a sigma factor, during non-stressful conditions and both factors play a role in regulating stress response (Song, et al., 2003). Poole (2012) had also associated antibiotic resistance to dysregulated stress response in bacteria. However, the detailed mechanism on how *rshA* mutations and its sigma factor, SigH cause tigecycline resistance in *M. abscessus* remains elusive.

5.2 Bacterial Adenylate Cyclase Two Hybrid (BACTH) system demonstrated disrupted RshA-SigH interaction in CL5A

During non-stressful conditions, RshA functions as a negative regulator which suppresses the SigH-dependent transcription (Song, et al., 2003). The interaction

between RshA and SigH was shown to be disrupted by redox stress, elevation in temperature or mutation in the conserved HXXXCXXC motif in *M. tuberculosis* (Song, et al., 2003). However, the interaction between these two proteins has not been studied in *M. abscessus* yet.

Thus, the BACTH system designed to study protein-protein interaction was used in this study to analyse the interaction between RshA and SigH from *M. abscessus* ATCC 19977 and CL5A. Interestingly, this system was also used in the study by Song, et al. (2003) to demonstrate the interaction between *M. tuberculosis* RshA and SigH, which could interact *in vivo*.

As anticipated, a slower development of blue colour was observed in the *E. coli* reporter strain BTH101 which was co-transformed with pKT25-*MAB_3543c*^{wt} and pUT18-*MAB_3542c*^{CL5Amut} (Figure 4.3). This finding suggested that the *rshA* mutation in CL5A could disrupt the interaction between RshA and SigH. RshA functions as an anti-sigma factor that inhibits the sigma factor SigH under normal conditions. In the presence of stress, the bond between these two proteins would be disrupted. The findings in this present study showed that, in the absence of external stressors, the interaction between RshA and SigH could be disrupted by the *rshA* mutation in CL5A

5.3 RT-qPCR verified the upregulation of *sigH* in CL5A and CL6A

Following the previous observation (Figure 4.3), in which the interaction between the two proteins was disrupted due to the *rshA* mutations, SigH would be released, increasing the *sigH* transcription.

To verify this, RT-qPCR analysis on CL5A and CL6A was performed to quantify the mRNA levels of the *sigH* gene. As anticipated, the results demonstrated that the *sigH* gene was significantly upregulated in both tigecycline-resistant mutants. The results of this study confirmed that *rshA* mutations affected its function to inhibit SigH, allowing the release SigH to bind to the RNA polymerase, and auto-upregulate itself (Song, et al., 2003), even in the absence of external stressors.

This was congruent with the observation by (Song, et al., 2003) that the interrupted RshA-SigH interaction frees SigH to auto-upregulate itself as well as other downstream stress response genes.

Hence, if RshA mutations were to lead to an enhanced mycobacterial stress response, the bacteria which possess these mutations would have a selective advantage in harsh natural environments. It may be interesting to study the response of these RshA mutants to experimentally induced stresses both *in vitro* and *in vivo*.

Thus, if bacterial strains like these laboratory-derived mutants were to be present in the environment, horizontal transfer of the genetic determinant of tigecycline resistance to other bacteria could occur over time to cause widespread tigecycline resistance as Wintersdorff, et al. (2016) stated that antimicrobial resistance could be acquired via horizontal gene transfer of antibiotic resistance genes.

5.4 Efflux-encoding genes in the tigecycline-resistant mutants, CL5A and CL6A

RNA sequence analysis of 7C revealed the upregulation of three genes (*MAB_1395, MAB_1396* and *MAB_1299c*) annotated as putative drug/multidrug transporters (Ng, et al., 2020). As these three efflux genes were upregulated alongside the *rshA* mutation in 7C, it is likely for these efflux genes to be involved in the mechanism of tigecycline resistance in *M. abscessus* (Ng, et al., 2020). Hence, these three efflux-encoding genes were selected for further characterisations.

The RT-qPCR analysis revealed that these three efflux genes were generally upregulated in CL5A and CL6A. This insinuated that the efflux mechanism may be the missing piece linking the *rshA* mutants and their eventual tigecycline resistance phenotype as evidenced by the contribution of efflux pumps in antimicrobial resistance in other bacteria, including tigecycline. This is not surprising as efflux pumps had been documented to be linked to tigecycline resistance in Gram-negative bacteria and occasionally, Gram-positive bacteria

as well (Sun, et al., 2014b). Efflux genes were also associated with clarithromycin resistance in *M. abscessus* (Vianna, et al., 2019; Guo, et al., 2020).

5.5 Overexpression of the three efflux-encoding genes failed to induce resistance to tigecycline in *M. abscessus*

As it was suspected that the three efflux genes might have contributed to tigecycline resistance in the *rshA* mutants, these genes (*MAB_1395*^{wt}, *MAB_1396*^{wt} and *MAB_1299c*^{wt}) were overexpressed in the parental ATCC 19977 strain.

Unfortunately, the overexpression of the genes in the wt ATCC 19977 did not show an increase in tigecycline resistance as tested by the agar disc diffusion method (Table 4.5). Hence, it appeared that the overexpression of efflux genes was a consequence of *rshA* mutations but was not, on its own, the cause of tigecycline resistance.

It is known that efflux pumps often work in synergy with other mechanisms of resistance. It would be interesting for future studies to further explore the role of efflux pumps in the mechanism of tigecycline resistance in *M. abscessus*.

5.6 Limitations

This study showed an important role for *rshA* mutations in tigecycline resistance in *M. abscessus*. It is postulated that the resistance is mediated by an increase in SigH production caused by *rshA* mutations. Unfortunately, it is beyond the scope of this study and the tenure of a Master candidature to identify the mechanisms downstream to the *sigH* upregulation that lead to tigecycline resistance.

The findings in this current study were based on the characterisation of the tigecycline-resistant laboratory-derived mutants, CL5A and CL6A. No clinical strains of *M. abscessus* were screened for *rshA* mutations. Widening the search for *M. abscessus* strains from patients who do not respond to tigecycline treatment might lead to the discovery of more tigecycline resistance determinants in these pathogens.

CHAPTER 6

CONCLUSION

The original contribution in this study is the demonstration that the K71E and insertional mutations in the *rshA* gene can interrupt the interaction between RshA and SigH, two transcriptional regulators of physiological stress response in mycobacteria. The disrupted interaction is believed to lead to the overexpression of *sigH* which contributes to the phenotypic resistance to tigecycline in *M. abscessus*.

Taken together, the mechanism of tigecycline resistance in CL5A and CL6A appears to be much of the same as the previously reported mechanism in the mutant 7C. In all the mutants, *rshA* mutations (in 7C, CL5A and CL6A) disrupt the *in vivo* interaction of RshA and SigH to cause an upregulation of *sigH*. However, the downstream events linking the *sigH* upregulation and tigecycline resistance are still obscure. While it is well-known that sigma factors regulate antibiotic resistance in many bacteria by different mechanisms such as reducing the expression of outer membrane proteins or increasing multidrug efflux pumps, it has also been noted that the same sigma factor may have opposite effects on antimicrobial resistance in different bacteria (Woods and McBride, 2017). Further work will be required to understand how SigH protects *M. abscessus* against the tigecycline onslaught.

As tigecycline is still presently one of the most effective treatments for *M. abscessus* infections, the results from this study will hopefully contribute to a better understanding of tigecycline resistance in *M. abscessus* and hence, prolong the lifespan of tigecycline use in the clinical treatment against infections caused by this organism.

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APPENDIX A

The preparation of culture media used in this study

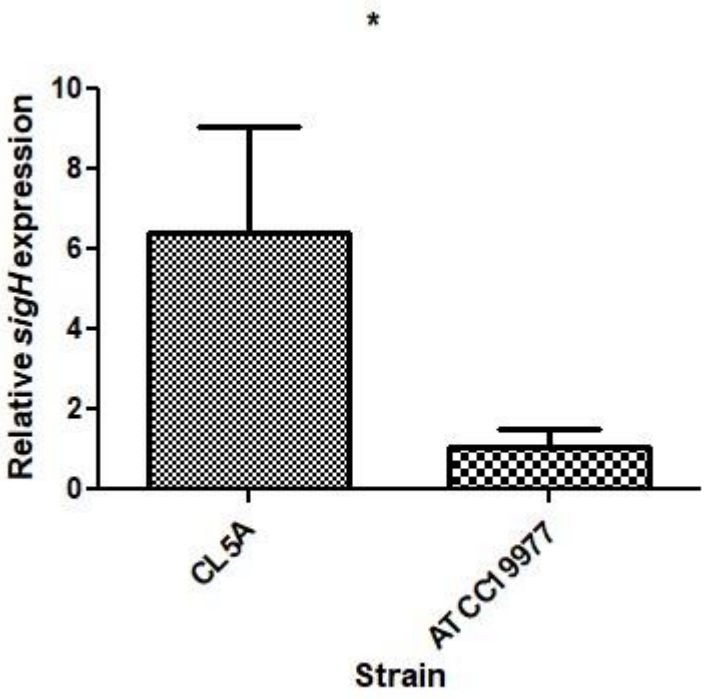
Medium	Recipe (per L)	Remarks
Cation-adjusted Mueller-Hinton agar	Not applicable	Pre-poured culture media
Cation-adjusted Mueller-Hinton broth	22g of powder	Not applicable
Luria-Bertani agar	40g of powder	Not applicable
Luria-Bertani broth	25g of powder	Not applicable
Middlebrook 7H9 broth	4.7 g of powder 5 mL of glycerol 2.5 mL of Tween 80 100 mL of Middlebrook ADC enrichment[1]	Not applicable
Middlebrook 7H10 agar	19 g of powder 5 mL of glycerol 100 mL of Middlebrook OADC enrichment[1]	Not applicable

Other than the pre-poured culture media, the agar and broth used in this current study were prepared by adding a final volume of 1 L of distilled water into the dehydrated culture media, which was in the form of powder. The mixture was sterilised by autoclaving at 121 ℃ for 20 minutes.

[1]As it is heat-labile, this ingredient was only added in after autoclaving when the media had cooled down to an approximate of 55 ℃.

APPENDIX B

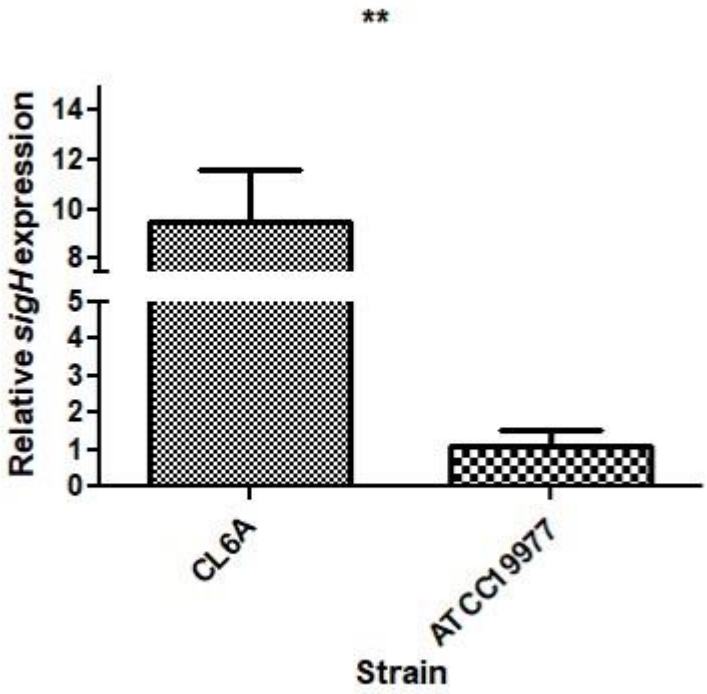
Relative gene expression of *MAB_3543* (*sigH*) in CL5A and ATCC 19977



The *sigH* gene was significantly upregulated in CL5A as compared to ATCC 19977.

APPENDIX C

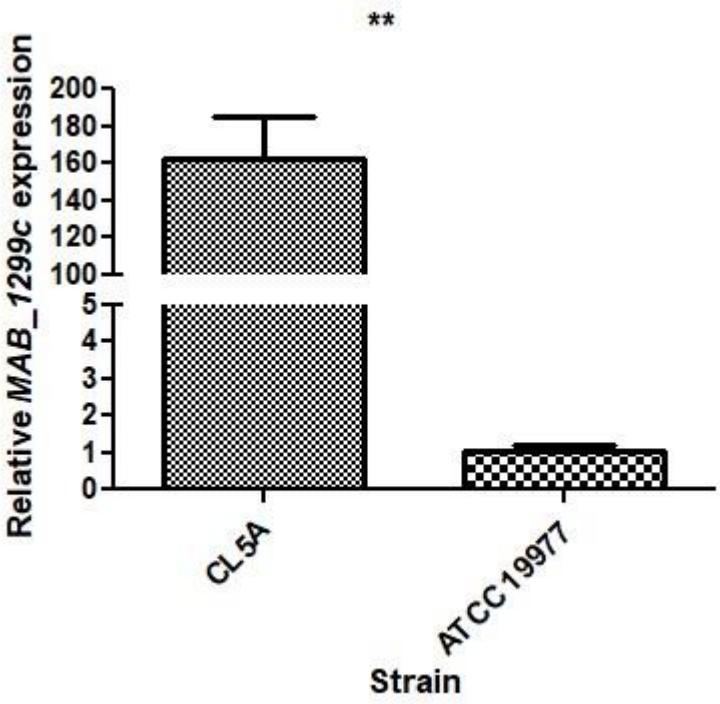
Relative gene expression of *MAB_3543* (*sigH*) in CL6A and ATCC 19977



The *sigH* gene was significantly upregulated in CL6A as compared to ATCC 19977.

APPENDIX D

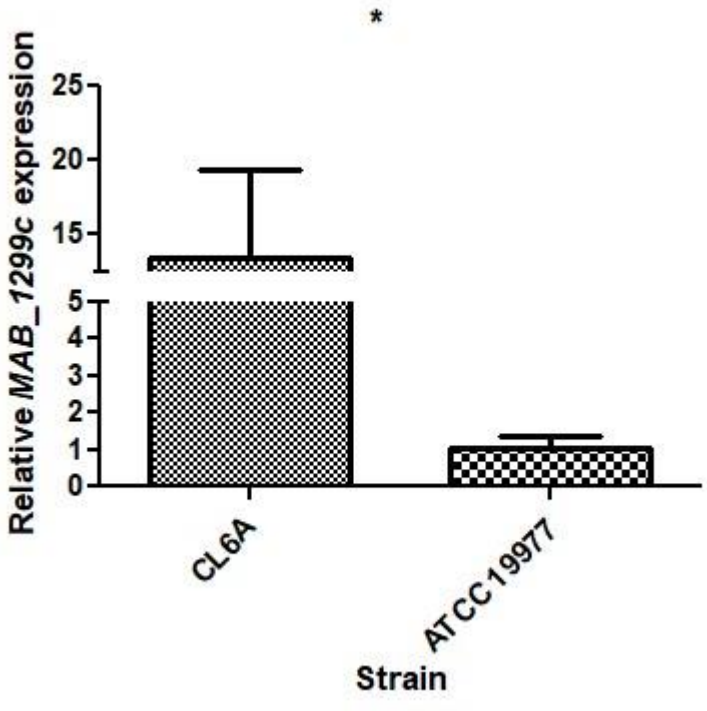
Relative gene expression of *MAB_1299c* in CL5A and ATCC 19977



MAB_1299c was significantly upregulated in CL5A as compared to ATCC 19977.

APPENDIX E

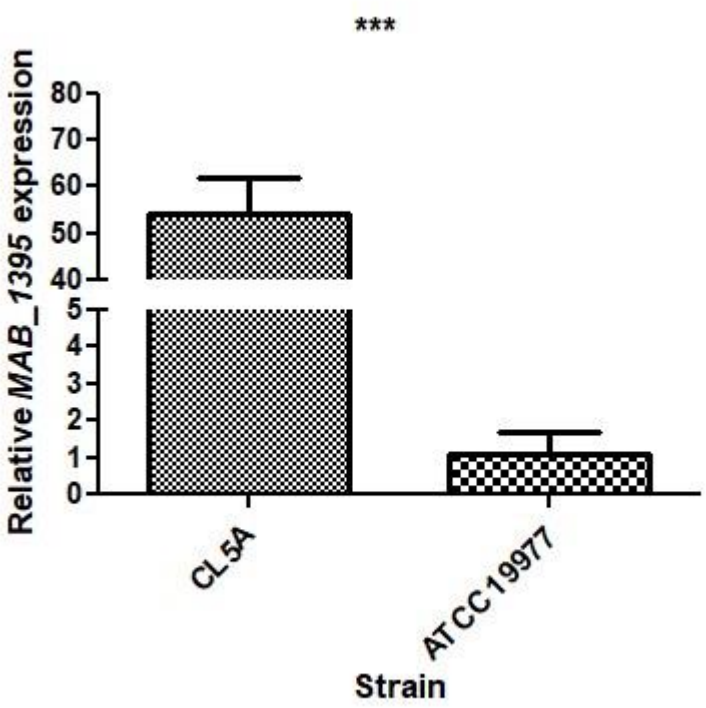
Relative gene expression of *MAB_1299c* in CL6A and ATCC 19977



MAB_1299c was significantly upregulated in CL6A as compared to ATCC 19977.

APPENDIX F

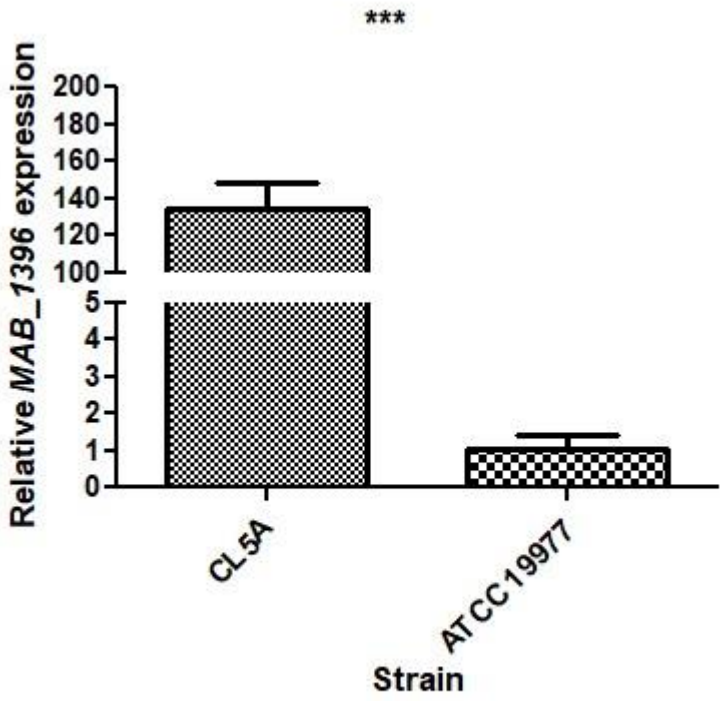
Relative gene expression of *MAB_1395* in CL5A and ATCC 19977



MAB_1395 was significantly upregulated in CL5A as compared to ATCC 19977.

APPENDIX G

Relative gene expression of *MAB_1396* in CL5A and ATCC 19977



MAB_1396 was significantly upregulated in CL5A as compared to ATCC 19977.

LIST OF PUBLICATIONS AND PAPER PRESENTED

This study had produced one manuscript which has been accepted for publication by the Journal of Medical Microbiology, a Microbiology Society journal. On top of that, a poster was presented in the 2021 International Congress of Pathology and Laboratory Medicine (ICPaLM) and the abstract was published in The Malaysian Journal of Pathology.

The reference for the abstract published is as follows:

Aw, K.M., Ng, H.F., Ngeow, Y.F. and Zin, T., 2021. The up-regulation of three efflux genes may be associated with the *MAB_3542c* mutation which confers tigecycline resistance in *Mycobacteroides abscessus*. *The Malaysian Journal of Pathology*, [online] 43(1), p.190. Available at: <http://www.mjpath.org.my/2021/v43n1/abstracts-ICPALM.pdf> [Accessed 6 June 2022].

The following is the email of acceptance by the Journal of Medical Microbiology:

Manuscript number: JMM-D-22-00060R1

Title: RshA mutations contributing to tigecycline resistance in *Mycobacteroides abscessus*

Dear Dr Yun Fong Ngeow,

Authors: Kar Men Aw, Hien Fuh Ng, Yun Fong Ngeow, Thaw Zin

I am pleased to tell you that your article has now been accepted for publication in *Journal of Medical Microbiology*. The Editorial Office will now check that

they have all the files needed for publication and contact you if anything else is required.

If your institution is planning on issuing a press release about this research, please inform us so that we can work with your press team to maximise its impact.

The Microbiology Society journals encourage all authors to deposit the accepted, non-copyedited or typeset version of their article in a subject or institutional repository on the day of publication. This is known as Green Open Access. For more information, see our Open Access Policy page: www.microbiologyresearch.org/oa-policy#2.

Please let us know about your experience with our journals by completing this 2-minute survey: www.surveymonkey.co.uk/r/789JDDC

Congratulations again and thank you for submitting your paper to *Journal of Medical Microbiology*. I hope that you will consider us for future submissions.

Kind regards,

Dr Efthymia Petinaki

Editor, *Journal of Medical Microbiology*

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