SELECTION AND CHARACTERIZATION OF A TIGECYCLINE-RESISTANT MUTANT OF *MYCOBACTERIUM ABSCESSUS* TO IDENTIFY POSSIBLE RESISTANCE DETERMINANTS

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By

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ABSTRACT

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NG HIEN FUH

Rapid-growing mycobacteria (RGM) are highly susceptible to tigecycline. In particular, this antibiotic has shown good *in vitro* and *in vivo* activities against *Mycobacterium abscessus* (*Mab*), a clinically important species of RGM known for its multidrug resistance phenotype. The genetic determinants of tigecycline resistance in this bacterium are not well-elucidated. In the present study, a laboratory-derived tigecycline-resistant mutant of *Mab* ATCC 19977, named as 7C, was selected and characterized to identify possible mechanisms of tigecycline resistance. Based on the principles of the Luria-Delbrück experiment, resistant mutants were selected from *Mab* ATCC 19977 (tigecycline minimum inhibitory concentration [MIC]: 0.25 mg/L; imipenem MIC: 8 mg/L) on agar with increasing concentrations of tigecycline. The most resistant mutant, 7C, was selected for further characterizations, including genotyping, screening for cross resistance, study of fitness cost, whole-genome sequencing (WGS), RNA sequencing (RNA-Seq), and time-kill assays. Biological verification of WGS findings were carried out through cloning and transformation experiments. Compared to the wild type, 7C demonstrated resistance to tigecycline (MIC: 2 mg/L) as well as cross-resistance to imipenem (MIC: ≥32 mg/L), and had a slightly retarded growth rate. Using WGS, 7C was

found to harbor a T151C mutation in the *MAB_3542c* gene. Complementation with the wild-type *MAB_3542c* reverted 7C back to the tigecycline-susceptible (MIC: 0.125 mg/L), imipenem-susceptible (MIC: 4 mg/L), and faster-growing phenotypes, suggesting that these phenotypes of 7C were caused by the point mutation in *MAB_3542c*, which encodes an RshA-like protein. In *M. tuberculosis*, RshA is an anti-sigma factor that negatively regulates the heat/oxidative stress response mechanisms. RNA-Seq analysis of 7C revealed that this mutation may dysregulate the stress-response pathways which have been shown, in the previous studies, to be linked to antibiotic resistance. In line with the RNA-Seq findings, 7C demonstrated an improved survival against heat shock, suggesting that dysregulated stress response may be a possible mechanism of tigecycline resistance in *Mab*. Another interesting observation from RNA-Seq analysis was the down-regulation of ribosomal protein-encoding genes. This highlights the possibility of ribosomal conformation changes which could negatively affect the binding of tigecycline to its target, leading to phenotypic resistance. Lastly, it was demonstrated that transient tigecycline resistance can be induced in the wild-type ATCC 19977 by elevated temperature, reiterating the possible role played by the stress response in tigecycline resistance. In general, the *MAB_3542c* mutation may represent a novel determinant of tigecycline resistance. In a broader perspective, the elucidation of tigecycline resistance mechanisms in *Mab* will contribute valuable information to the development of novel antibiotics so essential for the global struggle to mitigate multiple drug resistance in bacterial pathogens.

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APPROVAL SHEET

This dissertation/thesis entitled "**SELECTION AND CHARACTERIZATION OF A TIGECYCLINE-RESISTANT MUTANT OF *MYCOBACTERIUM ABSCESSUS* TO IDENTIFY POSSIBLE RESISTANCE DETERMINANTS"** was prepared by NG HIEN FUH and submitted as partial fulfillment of the requirements for the degree of Doctor of Philosophy (Medical Science) at Universiti Tunku Abdul Rahman.

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SUBMISSION OF THESIS

It is hereby certified that NG HIEN FUH (ID No: 16UMD04174) has completed this thesis entitled "SELECTION AND CHARACTERIZATION OF A TIGECYCLINE-RESISTANT MUTANT OF *MYCOBACTERIUM ABSCESSUS* TO IDENTIFY POSSIBLE RESISTANCE DETERMINANTS" under the supervision of Prof. Ngeow Yun Fong (Supervisor) from the Department of Pre-clinical Sciences, Faculty of Medicine and Health Sciences, and Dr. Thaw Zin (Co-Supervisor) from the Department of Pre-clinical Sciences, Faculty of Medicine and Health Sciences.

I understand that University will upload softcopy of my thesis in pdf format into UTAR Institutional Repository, which may be made accessible to UTAR community and public.

Yours truly,

_____

(NG HIEN FUH)

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.

Signature _____
(NG HIEN FUH)

Date _____

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LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
BCG	Bacille Calmette-Guérin
BLASTn	Nucleotide Basic Local Alignment Search Tool
BLASTp	Protein Basic Local Alignment Search Tool
CAMHA	Cation-adjusted Mueller-Hinton agar
CAMHB	Cation-adjusted Mueller-Hinton broth
CDS	Coding DNA sequence
CFU	Colony-forming unit
CLSI	Clinical and Laboratory Standards Institute
DAVID	Database for Annotation Visualization and Integrated Discovery
DEGs	Differentially expressed genes
EASE	Expression Analysis Systematic Explorer
ESBL	Extended-spectrum beta-lactamase
EUCAST	European Committee on Antimicrobial Susceptibility Testing
gDNA	Genomic DNA
GTP	Guanosine triphosphate
HIV	Human immunodeficiency virus
Mab	*Mycobacterium abscessus*
MATE	Multidrug and toxic compound extrusion
MaVA	*Mab* variable-number-tandem-repeat analysis
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant *Staphylococcus aureu*s
Mtb	*Mycobacterium tuberculosis*

NCBI	National Center for Biotechnology Information
NDM-1	New Delhi metallo-beta-lactamase-1
NGS	Next-generation sequencing
NTC	No-template control
NTM	Non-tuberculous mycobacteria
OD600	Optical density at 600 nm
PRSP	Penicillin-resistant *Streptococcus pneumoniae*
RGM	Rapid-growing mycobacteria
RIN	RNA Integrity Number
RNA-Seq	RNA sequencing
RND	Resistance-nodulation-cell division
ROS	Reactive oxygen species
rRNA	Ribosomal RNA
RT-qPCR	Reverse-transcriptase real-time PCR
SGM	Slow-growing mycobacteria
SOC	Super optimal broth with catabolite repression
TSB	Tryptic soy broth
UV	Ultraviolet
VRE	Vancomycin-resistant enterococci
WGS	Whole-genome sequencing
wt	Wild type

CHAPTER 1

INTRODUCTION

1.1 Background

Mycobacterium abscessus (*Mab*) is a clinically important member of the non-tuberculous mycobacteria (NTM). It is known to be highly resistant to multiple classes of antibiotics (Brown-Elliott & Wallace 2002; Nessar et al. 2012). Tigecycline is one of the few remaining drugs that are still active against this species complex (Nessar et al. 2012). Unfortunately, clinically resistant strains have been reported in recent years (Broda et al. 2013; Ananta et al. 2018). An understanding of resistance mechanisms is essential in the adoption of appropriate measures to prevent, delay, or circumvent the further escalation of resistance that will shorten the useful lifespan of this antibiotic.

1.2 Problem statement and hypothesis

The mechanism of tigecycline resistance in *Mab* is not well understood. Hence, the research question for this study was: What are the genetic determinants of tigecycline resistance in *Mab*?

The hypothesis made was: The development of tigecycline resistance may involve hitherto unreported genetic determinant(s) in *Mab*.

1.3 Objectives

The principal objective of this study was to identify possible genetic determinant(s) of tigecycline resistance in *Mab*. The specific objectives were: (a) to select and characterize a laboratory-derived mutant of *Mab* expressing resistance to tigecycline; (b) to correlate genotypic and phenotypic tigecycline resistance; (c) to study the possible effects of resistance determinant(s) through transcriptomic analysis.

CHAPTER 2

LITERATURE REVIEW

2.1 Tetracyclines

Tetracyclines are antibiotics that act as protein synthesis inhibitors by blocking the charged tRNA from entering the ribosomal acceptor (A) site. This family of antibiotics is marked by a linear fused tetracyclic nucleus, to which various functional groups are attached (Chopra & Roberts 2001). Tetracycline molecules exhibit activity against a broad spectrum of pathogenic bacteria, including members of the gram-positive and gram-negative groups and atypical organisms such as mycoplasmas, chlamydiae, and rickettsiae (Chopra & Roberts 2001). In addition, tetracyclines are also active against eukaryotic, protozoan parasites (Chopra & Roberts 2001). Owing to their reversible association with the ribosome, tetracyclines are generally bacteriostatic in nature (Chopra et al. 1992). Principal members of tetracycline antibiotics are summarized in Table 2.1.

Table 2.1 Principal members of tetracyclines.

Generation	Generic name	Chemical name
First	Chlortetracycline	7-Chlortetracycline
	Oxytetracycline	5-Hydroxytetracycline
	Tetracycline	Tetracycline
	Demethylchlortetracycline	6-Demethyl-7-chlortetracycline
	Rolitetracycline	2-*N*-Pyrrolidinomethyltetracycline
	Limecycline	2-*N*-Lysinomethyltetracycline
	Clomocycline	N-Methylol-7-chlortetracycline
Second	Methacycline	6-Methylene-5-hydroxytetracycline
	Doxycycline	6-Deoxy-5-hydroxytetracycline
	Minocycline	7-Dimethylamino-6-demethyl-6-deoxytetracycline
Third	Tigecycline	9-(*t*-butylglycylamido)-minocycline
Fourth	Omadacycline	(4S,4aS,5aR,12aS)-4,7-bis(dimethylamino)-3,10,12,12a-tetrahydroxy-9-((neopentylamino)methyl)-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide
	Eravacycline	7-fluoro-9-pyrrolidinoacetamido-6-demethyl-6-deoxytetracycline

The occurrence of tetracycline-resistant pathogens has dramatically reduced the usefulness of tetracyclines as therapeutic agents. The widespread use of tetracyclines has been suggested as the major factor contributing to an increase in the number of tetracycline-resistant bacteria (Chopra & Roberts 2001). In particular, the use of tetracyclines in animal feeds as a growth promoter poses a significant risk for the selection of resistant bacteria (Swann 1969). This is because the dosage of antibiotics used in animal husbandry is at a continuous, sub-therapeutic level, as compared to the short-term, higher therapeutic level used to treat human or animal infections. Disconcertingly,

there is ample scientific evidence to suggest that antibiotic-resistant pathogens can be transmitted from livestocks to humans (Mølbak et al. 1999; Wegener 2012).

The onset of tetracycline resistance has frequently been associated with the acquisition of genes encoding efflux or ribosomal protection proteins through horizontal gene transfer. Efflux pumps are membrane proteins which pump or remove antibiotics out of the cell by using the adenosine triphosphate (ATP) or the proton motive force as energy source, leading to a reduced cytoplasmic concentration of antibiotics. Genes which code for the efflux proteins, such as *tetA, tetC, tetE, tetG,* and *tetH*, have been reported to be a major determinant of tetracycline resistance (Chopra & Roberts 2001). On the other hand, ribosomal protection represents another important mechanism in promoting tetracycline resistance by protecting the bacterial ribosome from the binding of the tetracycline. Two of the most well-studied ribosomal protection proteins are TetO and TetM, which have been shown to be able to dislodge tetracycline from the bacterial ribosome in a guanosine triphosphate (GTP)-dependent manner (Burdett 1996; Trieber et al. 1998). The dislodge of tetracycline liberates the ribosome from the inhibitory effect of the drug, allowing the aminoacyl-tRNA to bind to the ribosomal A site so that protein synthesis can continue and conferring tetracycline resistance in the process (Connell et al. 2003). Many of the efflux- and ribosomal protection protein-encoding genes are frequently found in mobile genetic elements, which may have accelerated their spread throughout the eubacteria via lateral-gene transfer (Chopra & Roberts 2001). Enzymatic modification represents the rarest type of

tetracycline-resistance mechanism. The TetX is the only known tetracycline-inactivating enzyme to date (Speer et al. 1991) (see section 2.1.1). Tetracycline resistance can also arise through mutations which cause changes in the regulation of the intrinsic efflux systems (Levy 1992) or the 16S ribosomal RNA (rRNA) (Ross et al. 1998).

2.1.1 Tigecycline

Tigecycline is the first and only clinically available drug in a new class of tetracycline antibiotics known as the glycylcycline. It is a derivative of minocycline, with a N,N-dimethyglycylamido moiety attached to the 9' carbon on the tetracycline four-ringed skeleton (Townsend et al. 2006) (Figure 2.1). Similar to other tetracyclines, tigecycline is a bacteriostatic antimicrobial agent which impedes translation by binding to the A site of the 30S ribosomal subunit (Noskin 2005). The protein-synthesis inhibitory activity of tigecycline has been shown to be 3- and 20-fold more potent than that of minocycline and tetracycline, respectively (Olson et al. 2006). The ability of tigecycline to evade two common mechanisms of tetracycline resistance, active efflux and ribosomal protection (Noskin 2005), is generally attributed to its bulky side chain (Schedlbauer et al. 2015). In addition, a molecular modeling study showed that tigecycline has additional interaction with ribosomal H34 and H18 nucleotides, in comparison to tetracycline and minocycline (Olson et al. 2006). These characteristics have been speculated to help the drug to bind (a) in a different orientation and (b) with greater affinity than tetracycline (Bauer et al. 2004), preventing recognition by the Tet efflux transporters and ribosomal

protection proteins (Rasmussen et al. 1994; Sun et al. 2013).

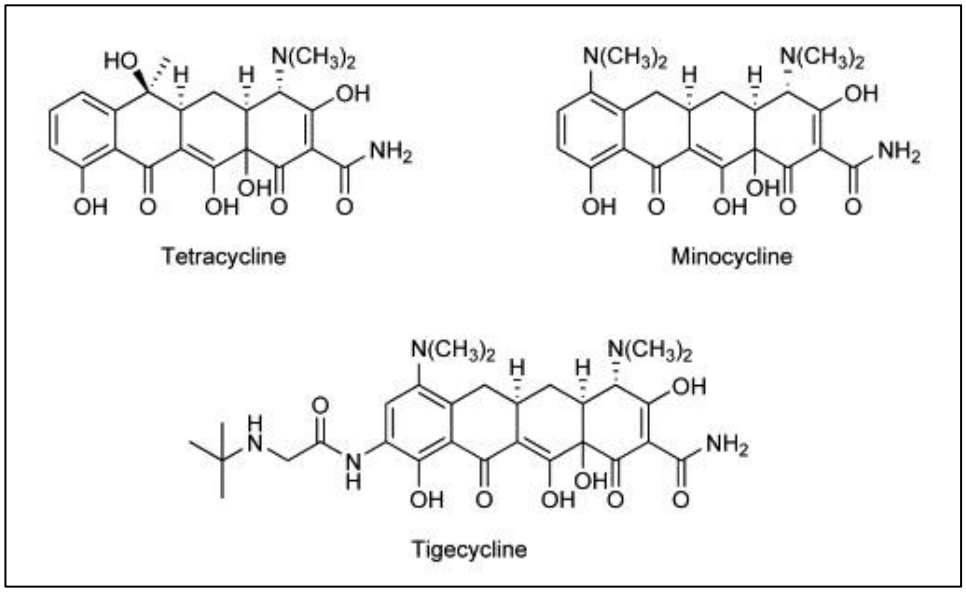

Figure 2.1: Chemical structures of tetracycline, minocycline, and tigecycline.

Tigecycline displays a broad spectrum of antibacterial activity. It is active against important pathogens like methicillin-resistant *Staphylococcus aureu*s (MRSA), penicillin-resistant *Streptococcus pneumoniae* (PRSP), vancomycin-resistant enterococci (VRE), and extended-spectrum beta-lactamase (ESBL) producers (Noskin 2005). Furthermore, tigecycline is one of the last-resort drugs (alongside colistin) to treat infections caused by bacteria expressing the New Delhi metallo-beta-lactamase-1 (NDM-1) that confers multidrug resistance (Kumarasamy et al. 2010).

Tigecycline resistance has emerged in recent years and is most frequently observed in gram-negative bacteria, mostly *Acinetobacter baumannii* and members of the Enterobacteriaceae (Sun et al. 2013). The

decreased susceptibility to tigecycline in these clinically important organisms has generally been attributed to the over-expression of resistance-nodulation-cell division (RND)-type transporters, such as the AcrAB efflux pumps (Sun et al. 2013). In addition, mutations located in genes encoding the ribosomal protein S10 (Villa et al. 2014), a SAM-dependent methyltransferase (Chen et al. 2014), the acyl-sn-glycerol-3-phosphate acyltransferase (Li et al. 2015), and proteins involved in the lipopolysaccharide core biosynthesis (Linkevicius et al. 2013) have also been associated with tigecycline resistance in gram-negative organisms. A novel mechanism of tigecycline resistance is the TetX-mediated oxygen-dependent destruction of the drug (Moore et al. 2005). Ironically, TetX was originally found in *Bacteroides*, which is a genus of obligate anaerobes. Hence, this enzyme is unlikely to confer tigecycline resistance to its natural host. Disconcertingly, a study published in 2013 reported the isolation of multidrug-resistant, *tetX*-containing, and aerobic strains of bacteria from clinical samples collected in Sierra Leone (Leski et al. 2013), signifying a worrying trend of *tetX* dissemination through horizontal transfer. However, it is unclear whether these clinical isolates were resistant to tigecycline as this drug was not tested at the time of isolation. Tigecycline resistance has also been reported, albeit less frequently, in gram-positive organisms, like *Staphylococcus* spp. and *Streptococcus pneumoniae* (Sun et al. 2013). By characterizing laboratory-derived mutants, over-expression of the multidrug and toxic compound extrusion (MATE) family efflux pump MepA and mutations in ribosome-related genes were found to be the reasons behind resistance or decreased susceptibility to tigecycline in *S. aureus* and *S. pneumoniae*, respectively (McAleese et al. 2005; Lupien et al. 2015).

2.2 *Mycobacterium*

Mycobacterium is a genus assigned to the family of *Mycobacteriaceae*, under the phylum of Actinobacteria. Prominent features of mycobacteria include slow growth rate, acid-fastness, and genome with high guanine and cytosine content (Good & Shinnick 1998). Two of the most well-known species in this genus are *M. tuberculosis* (*Mtb*) and *M. leprae*, the causative agents for tuberculosis and leprosy (which is also known as Hansen's disease), respectively. Other members of this genus are NTM, which are also known as atypical mycobacteria. The major difference between NTM and their counterparts, *Mtb* complex and *M. leprae*, is that NTM are not obligate pathogens but are the environmental inhabitants (Primm et al. 2004). For this reason, NTM are sometimes referred to as environmental mycobacteria.

NTM can be found in a vast variety of environmental reservoirs, such as natural and municipal water, aerosols, and soil (Primm *et al.*, 2004). Under optimal conditions, NTM species which form visible colonies within a week are known as rapid-growing mycobacteria (RGM) while those requiring longer periods are known as slow-growing mycobacteria (SGM) (Stahl & Urbance 1990). It is important to note that the growth rate of the RGM is still substantially slower than most other bacteria. The slow growth rate of mycobacteria could be attributable to impermeability of their lipid-laden cell envelope to certain hydrophilic nutrients (Primm et al. 2004), high energy cost for synthesizing long-chain mycolic acids (Primm et al. 2004), and low copy number of the *16S rRNA* genes in their genomes (Bercovier et al. 1986).

When one or more predisposing host conditions are met, NTM can become opportunistic pathogens. Risk factors of NTM infection include pre-existing pulmonary conditions such as chronic obstructive pulmonary disease, cystic fibrosis, or tuberculosis, and impaired immunity rendered by the human immunodeficiency virus (HIV) infection, the usage of immunosuppressive drugs, or leukemia (Griffith et al. 2007). Some examples of diseases caused by NTM are Buruli ulcer, a cutaneous infection caused by *M. ulcerans*, and pediatric cervical lymphadenitis, an infection caused by *M. avium* complex and *M. scrofulaceum* (Griffith et al. 2007). Unlike *Mtb*, human-to-human transmission of NTM is fairly uncommon (Griffith et al. 2007), although several studies have provided evidence that transmission of certain NTM species is possible under ideal circumstances (Bryant et al. 2013; Ricketts et al. 2014). In addition to causing diseases, some also believe that exposure to NTM in the environment may reduce the level of protection afforded by the Bacille Calmette-Guérin (BCG) vaccine against tuberculosis (Stanford et al. 1981; Weir et al. 2006; Poyntz et al. 2014).

2.2.1 *Mycobacterium abscessus*

Discovered in the 1950s (Moore & Frerichs 1953), *Mab* is one of the RGM that causes a wide spectrum of infections in humans, including but not limited to pulmonary and soft-tissue infections, and disseminated infections (Griffith et al. 2007). More importantly, it is notorious for its resistance to multiple antibiotics, mediated through its intrinsic features (Table 2.2) or through chromosomal mutations that arise under the selective pressure of

antibiotic use (Table 2.3). Thus, *Mab* poses a major threat to clinical management and public health as treatment options for this bacterium are very limited.

Certain intrinsic features of *Mab* are inducible in nature. Genes encoding the enzymes, Erm(41) (a methyltransferase which modifies the target of macrolides) and MabTetX (a tetracycline-modifying enzyme), have been shown to be induced by exposure to macrolides and tetracyclines, respectively (Nash et al. 2009; Rudra et al. 2018). To date, the molecular mechanisms of tigecycline resistance in *Mab* remain largely unknown. In 2017, Pryjma et al. found *whiB7* (*MAB_3508c*) to be associated with reduced tigecycline susceptibility in *Mab* (Table 2.2); the deletion of the WhiB7-encoding gene caused a 4-fold decrease in the minimum inhibitory concentration (MIC) of tigecycline (from 0.8 to 0.2 mg/L).

On the other hand, mutations affecting the drug targets are commonly associated with antibiotic resistance. For example, mutations in *Mab* genes encoding 16S rRNA (key target of aminoglycosides) and 23S rRNA (key target of macrolides) are associated with resistance to aminoglycosides and clarithromycin, respectively (Wallace et al. 1996; Prammananan et al. 1998; Nessar et al. 2011). However, phenotypic resistance can also arise through alternative mechanisms (as indicated by the absence of mutations in the target genes). Occasionally, a mutation can alter the gene expression of the intrinsic features, leading to the development of antibiotic resistance. For instance, Richard et al. (2018) demonstrated that point mutations in the TetR-encoding

gene (*MAB_2299c*), which conferred resistance to bedaquiline and clofazimine to *Mab*, were linked to the up-regulation of genes encoding the MmpS/MmpL efflux system. Subsequent deletion of these two efflux-encoding genes abolished the resistance phenotype of the *MAB_2299c* mutant. This implies that the *MAB_2299c* mutations bestowed the bedaquiline and clofazimine resistance upon *Mab* through over-expression of the MmpS/MmpL efflux proteins. In a broader perspective, Richard et al. (2018) demonstrated that it is possible for *Mab* to develop resistance to these two drugs. This is especially worrisome as bedaquiline and clofazimine are two of the few remaining drugs that are still active against this bacterium (Nessar et al. 2012).

Acquired resistance mediated by mobile genetic elements, such as plasmids and transposons, is relatively rare among mycobacteria (Nessar et al. 2012). Thus far, only one study has documented the plasmid-mediated resistance to kanamycin in a clinical strain of *Mab* subspecies *bolletii* (Matsumoto et al. 2014).

In 2006, the *Mab* species has been subdivided into three closely related subspecies, namely *Mab* subsp. *abscessus*, *Mab* subsp. *massiliense* (Adékambi et al. 2004), and *Mab* subsp. *bolletii* (Adékambi et al. 2006), hereafter referred to as *Mab*, *M. massiliense* and *M. bolletii* respectively. Both *M. massiliense* and *M. bolletii* were briefly combined into a single subspecies in 2011 (Leao et al. 2011), but were separated again in 2016 as new evidence suggested that the three subspecies are genetically distinctive from each other (Tortoli et al. 2016). The differentiation of these three subspecies is not straightforward, as they

share ribosomal sequences, and single-gene PCR-sequencing approaches are unable to distinctively classify clinical strains to the subspecies level (Macheras et al. 2009; Zelazny et al. 2009). Nonetheless, these subspecies may differ from each other in terms of their antibiotic-resistant phenotype/genotype (Bastian et al. 2011), their variable-number tandem-repeat (VNTR) pattern (Wong et al. 2012), and their mass spectra (Fangous et al. 2014), which can be exploited for subspecies classification for the *Mab* complex.

Table 2.2: Intrinsic features that confer reduced susceptibility or resistance to antibiotics in *Mycobacterium abscessus*.

Target antibiotic(s)	Intrinsic feature	Gene	Mechanism of resistance	Reference
Amikacin	A putative acetyltransferase	*MAB_3168c*	Drug-modifying enzyme	Tsai et al. 2013
Amikacin, clarithromycin, tigecycline	WhiB7	*MAB_3508c*	Transcriptional activator	Pryjma et al. 2017
Aminoglycosides	AAC(2') Eis2 MAB_2385	*MAB_4395* *MAB_4532c* *MAB_2385*	Drug-modifying enzyme	Rominski, Selchow, et al. 2017; Dal Molin et al. 2017
Beta-lactams	Bla(Mab)	*MAB_2875*	Drug-modifying enzyme	Soroka et al. 2014
Clofazimine, bedaquiline	MmpS/MmpL	*MAB_2300*/*MAB_2301*	Efflux	Richard et al. 2018
Macrolides	Erm(41)	*MAB_2297*	Target-modifying enzyme	Nash et al. 2009
Rifamycins	Arr_Mab	*MAB_0951*	Drug-modifying enzyme	Rominski, Roditscheff, et al. 2017

Table 2.2 (continued): Intrinsic features that confer reduced susceptibility or resistance to antibiotics in *Mycobacterium abscessus*.

Target antibiotic(s)	Intrinsic feature	Gene	Mechanism of resistance	Reference
Tetracyclines	MabTetX	*MAB_1496c*	Drug-modifying enzyme	Rudra et al. 2018
Thiacetazone derivatives	MmpL/MmpS	*MAB_4382c*/*MAB_4383c*	Efflux	Halloum et al. 2017
Various antibiotics	Pmt	*MAB_1122c*	Limiting the permeability of cell envelope	Becker et al. 2017

Table 2.3: Mutations that confer antibiotic resistance to *Mycobacterium abscessus*.

Target antibiotic	Mutation(s)	Gene encoding	Reference
Aminoglycosides	A1408G, T1406A	16S rRNA	Prammananan et al. 1998; Nessar et al. 2012
Clarithromycin	A2058G, A2059C, A2059G	23S rRNA	Wallace et al. 1996
Clofazimine, bedaquiline	T119G, C276DEL, G541T, INS318A, T452C, G643A	TetR (MAB_2299c)	Richard et al. 2018

2.3 Tigecycline resistance in *Mycobacterium*

A recently published report demonstrated that tigecycline is a poor substrate of MabTetX and fails to induce the expression of this enzyme that has been shown to confer a high level of intrinsic resistance to tetracycline and doxycycline in *Mab* (Rudra et al. 2018). In addition, tigecycline also demonstrated good *in vivo* activity against *Mab* infections (Lerat et al. 2014; Oh et al. 2014). Interestingly, tigecycline has shown synergistic activities with other antibiotics against *Mab in vitro* and *in vivo* (Huang et al. 2013; Lerat et al. 2014; Aziz et al. 2018). In 2014, Wallace et al. reported that, after receiving tigecycline-containing salvage regimens for more than a month, approximately 66 % of patients with *Mab* complex or *M. chelonae* infection (n = 38) showed clinical improvement. This led the authors to conclude that tigecycline may be a useful addition to other clinically available drugs in patients with these difficult-to-treat infections.

Several studies have shown that the RGM (including *Mab* complex) are generally susceptible to tigecycline *in vitro*. In United States of America, Wallace et al. (2002) found the MIC_{50} and MIC_{90} of tigecycline for 20 *Mab* complex isolates to be 0.12 and 0.25 mg/L, respectively. A study in Turkey which characterized seven isolates of *Mab* complex found the MIC range of tigecycline to be 0.25 mg/L to 1 mg/L (Cavusoglu et al. 2012). In Asia, the tigecycline MIC_{50} and MIC_{90} for 40 clinical isolates of *Mab* complex collected in Taiwan are 0.5 mg/L and 2 mg/L, respectively (Huang et al. 2010). Recently, Hatakeyama et al. (2017) reported that the MIC_{50} and MIC_{90} for 13 *Mab* complex isolates from Japan are 0.25 mg/L and 0.5 mg/L, respectively. MICs are generally interpreted according to the breakpoints established by the Clinical and Laboratory Standards Institute (CLSI) or the European Committee on Antimicrobial Susceptibility Testing (EUCAST). However, breakpoints for tigecycline have not been determined for mycobacteria. To further complicate matters, various studies have used different cutoff values to define sensitivity and resistance. Examples include the non-species related breakpoints (sensitive ≤0.25 mg/L, resistant >0.5 mg/L) proposed by the EUCAST (2018) and the ones used by Wallace et al. (2002) (resistant ≥8 mg/L) and Ananta et al. (2018) (low ≤1 mg/L, moderate = 2 mg/L, high ≥4 mg/L). This discrepancy was highlighted by Broda et al. (2013), a group of United Kingdom researchers who demonstrated that 81 % (according to the non-species related breakpoints set by EUCAST) or 4 % (according to the breakpoints used by Wallace et al [2002]) of *Mab* complex strains tested (n = 58) are resistant to tigecycline, depending on which breakpoint was used. These studies did not identify the *Mab* complex isolates to subspecies level and hence, their results might not

reflect the individual susceptibilities of the subspecies. This concern was addressed by a study in Korea that showed both *Mab* (*n* = 33) and *M. massiliense* (*n* = 24) to be 100% susceptible to tigecycline (according to the breakpoints used by Wallace et al [2002]) (Kim et al. 2015). However, the authors did not report the MIC range, MIC_{50}, or MIC_{90} of these isolates and hence, it is not possible to interpret the level of susceptibility with other breakpoints. A recent study in Thailand reported that 37.8 % of *M. massiliense* (*n* = 37) and 16.1 % of non-*massiliense* subspecies (*n* = 31) have high tigecycline MICs (≥4 mg/L) (Ananta et al. 2018).

On the other hand, SGM (MIC: 16 to >32 mg/L) and *Mtb* (MIC: 8 to 64 mg/L) are geneally resistant to tigecycline (Wallace et al. 2002; Coban et al. 2011).

2.4 Antibiotic resistance studies

The antimicrobial resistance status of a bacterium is generally defined by its genome and uncovering the molecular determinants behind the resistance may provide valuable insights into the development of new treatment modalities. Originally devised for the estimation of mutation rates in bacteria (Luria & Delbrück 1943), the Luria-Delbrück experiment has increasingly been used in modern days as a means to generate spontaneous mutants for the elucidation of antibiotic-resistance determinants (Nessar et al. 2011; Linkevicius et al. 2013; Chen et al. 2018). The theory of this groundbreaking

experiment is that, in bacteria, mutations occur in the absence of selection and at random, rather than being a response to selective pressure. The experimental set-up of a Luria-Delbrück experiment is relatively simple. In brief, a small number of wild-type (wt) cells are used to set up parallel cultures in a non-selective liquid medium. These parallel cultures are then grown to saturation and plated onto agar containing the antibiotic at a concentration higher than the MIC of the wt. The "mutant" colonies that appear after incubation are then subjected to further studies. Alternatively, mutants can also arise through serial passage of wt bacterium in broth with increasing concentrations of the drug (McAleese et al. 2005; Lupien et al. 2015; Richard et al. 2018); since the selection of resistance is conducted in liquid culture, single colonies should be isolated on a solid medium for subsequent characterization to avoid population heterogeneity. Mutants can also be generated through transposon mutagenesis, in which transposons are randomly integrated into genomic DNA to achieve gene knockout. By using this method, Phunpruch et al. (2013) identified that the *ksgA*, which encodes a 16S rRNA dimethyltransferase, is responsible for the intrinsic clarithromycin resistance observed in *Mtb*.

If the genes encoding the drug targets or the proteins involved in antibiotic adaptation, or the sequences of the transposon (for transposon mutants) are known, PCR-based analyses can be employed to identify the resistance determinants in these laboratory-derived mutants (Bergval et al. 2009; Nessar et al. 2011; Phunpruch et al. 2013). However, if an alternative and unknown mechanism is involved, it may pose a significant challenge to identify such genetic determinants. Fortunately, the advent of next-generation

sequencing (NGS) has enabled the identification of genetic variants on a genome- or transcriptome-wide scale. Millions of DNA moleculues or mRNA-derived cDNA molecules can be sequenced in parallel by NGS technologies, without the need of bacterial clones. Specifically, whole-genome sequencing (WGS) has enabled the inquiry of nearly every base in the genome with unprecedented sequencing depth, thus making the identification of all genetic variants possible within a single assay (Illumina 2017). In the past few decades, microarrays have been the instrument of choice for many transcriptomic studies in the field of antibiotic resistance (McAleese et al. 2005; Coyne et al. 2010; Suzuki et al. 2014; Händel et al. 2014). Nevertheless, this technology has a number of limitations, such as dependence upon existing knowledge about genome sequences and a limited dynamic range of detection because of saturation of signals and high background (Wang et al. 2009). RNA sequencing (RNA-Seq) is able to overcome these inherent limitations of microarrays by being able to detect hitherto unknown transcripts and to generate absolute rather than relative gene-expression measurements (Wang et al. 2009). Indeed, both WGS and RNA-Seq technologies can be utilized to delineate the relationship between the mutations, gene expression changes, and phenotypic resistance to antibiotics.

It is important to acknowledge that findings from WGS or RNA-Seq are observational in nature, and direct results from observational studies are problematic for causal inference (Glass et al. 2013). Nonetheless, these studies are useful in generating a hypothesis for further investigations. Experimental validation would then provide the evidence for interpretation in the subject

matter context. For hypothesis-driven studies (i.e. target genes or mutations are known), genome editing can be used to produce mutants with targeted modifications. For instance, Rudra et al. (2018) recently showed that the exposure of *Mab* to sub-inhibitory concentration of tetracycline significantly induced the gene expression of *MAB_1496c* (encoding MabTetX) (Table 2.2). By using phage recombineering, a mutant lacking of this gene was constructed. Remarkably, the deletion strain was 20-fold more sensitive to tetracycline and doxycycline than the wt bacterium. Interestingly, complementation of the mutant with the wt *MAB_1496c* gene restored the resistance phenotype, indicating that *MAB_1496c* is the major determinant of tetracycline resistance in *Mab*. Other than that, complementation has also been successfully implemented to confirm the resistance determinants in mutants generated by the Luria-Delbrück experiment, serial passage in broth with antibiotic, and transposon mutagenesis (Nessar et al. 2011; Phunpruch et al. 2013; Chen et al. 2014; Li et al. 2015). In addition, over-expression of target gene by altering the gene dosage can also be used to validate the findings from mutant characterization. For example, Halloum et al. (2017) demonstrated that the transformation of plasmids carrying the efflux *mmpS5/mmpL5* gene into the wt *Mab* strain leads to over-expression of the target gene and resistance to several thiacetazone compounds.

Other than laboratory-derived mutants, clinically resistant isolates of bacterial pathogens represent another vital resource for research on antibiotic resistance mechanisms. For example, sequencing of the *16S rRNA* gene from 26 clinical isolates of *Mab* revealed that 94 % of them harbor an A-to-G

substitution at position 1408, which was later found to be responsible for their aminoglycoside-resistant phenotypes (Prammananan et al. 1998). Nonetheless, the comparison of genotypes between resistant and sensitive clinical strains may not always be straightforward owing to their diverse genetic make-ups. The subdivision of *Mab* complex into different subspecies only further complicates matters. This issue can be circumvented by studying serial isolates recovered from the same individual (whereby the genetic backgrounds are often identical), of which resistance may be selected due to the antibiotic use for treatment. A study in 2015, which compared the genome sequences between serial isolates of multidrug-resistant *Mtb* from a patient, unveiled that mutations associated with resistance to various antibiotics and maintenance of persistent infection are accumulated over the course of 21 years (Meumann et al. 2015), thus showcasing the remarkable ability of *Mtb* to evolve inside the human host with chronic infection.

2.5 "Side effects" of antibiotic resistance

Compared to their susceptible counterparts, bacterial strains that have acquired resistance, whether through target alteration or other mechanisms, often have a reduced fitness, which is generally expressed as a decrease in growth rate, virulence, or transmission rate (Andersson 2006). This is because most resistance mechanisms impart a fitness cost onto the bacterium. For example, Lupien et al. (2015) showed that mutations in ribosome-related genes, which conferred tigecycline resistance, retarded the growth rate of *S.*

pneumoniae. These mutations might have imparted a fitness cost to the bacterium by affecting the protein-synthesis apparatus (ribosomes), causing the mutant to replicate at a slower rate in the absence of tigecycline. Other resistance mechanisms, such as the over-expression of efflux pumps, often impose a metabolic cost on the bacterial cells (Zampieri et al. 2017). Hence, it is widely believed that the fitness costs associated with most resistances may allow the susceptible and faster-growing bacteria to out-compete the resistant and slower-growing ones when antibiotic use is reduced. However, the fitness cost conferred by the resistance mutations can be partially or fully lifted by compensatory mutations without the loss of resistance (Andersson 2006). To further complicate matters, the rare occurrence of cost-free resistance mutations and genetic linkage of the resistance markers with other selected markers (i.e. the molecular determinant, selected under the selective pressure of commonly used drugs, confers cross-resistance to a less commonly used drug) have previously been reported (Reynolds 2000; Enne et al. 2004). These adaptations would greatly increase the likelihood of the antibiotic resistance determinants being retained through evolution. In line with this finding, a study published in 2008 reported that, although rifampin resistance is universally associated with a fitness cost in laboratory-derived mutants of *Mtb*, resistant strains isolated from the antibiotic-treated patients are often selected with no or low fitness reduction (Gagneux et al. 2006).

Sometimes, a single resistance mechanism, selected by the use of an antibiotic, is able to confer resistance to other related or non-related antibiotics. This phenomenon is known as cross-resistance. One example of cross-

resistance among related drugs is the A1400G mutation in the *16S rRNA* gene which confers high level of resistance to aminoglycoside antibiotics (kanamycin and amikacin) in *Mtb* (Alangaden et al. 1998). On the other hand, cross-resistance to other classes of antibiotic can be facilitated by multidrug efflux pumps that are capable of exporting different classes of antibiotics. For instance, a clofazimine-resistant mutant of *Mab* with over-expression of the *mmpS/mmpL* gene (encoding an efflux system) has been shown to be cross-resistant to bedaquiline (Richard et al. 2018).

2.6 The role of sigma and anti-sigma factors in antibiotic resistance

Sigma factors are essential for bacterial transcription. To initiate the RNA transcription, a core RNA polymerase binds to a sigma factor, which confers transcription specificity to certain promoters, to form the RNA polymerase holoenzyme (Woods & McBride 2017). This interaction with the sigma factor can direct the RNA polymerase to transcribe specific sets of genes, therefore providing a means for bacterial cells to regulate the expression of specific genes in response to certain conditions (Missiakas & Raina 1998). Under normal circumstances, sigma factors are sequestered, and therefore inhibited, by the anti-sigma factors; a signal (e.g. stress) would then trigger a proteolytic cascade which results in the release of the sigma factor, thus initiating the transcription (Helmann 1999).

Some sigma factors are involved in stress-response functions, which are known to compromise antimicrobial activities by promoting physiological changes in bacterial cells (Poole 2012). For instance, the deletion of gene encoding the SigB sigma factor, which governs the response to acidic, alkaline, osmotic, oxidative, and heat stress, has been shown to cause rifampin sensitivity in *Bacillus subtilis* (Bandow et al. 2002). In *Streptomyces coelicolor*, Yoo et al. (2016) demonstrated that SigR, which is known to regulate the thiol-oxidative stress response, confers resistance to translation-inhibiting antibiotics (chloramphenicol, erythromycin, lincomycin, and tetracycline). On the other hand, anti-sigma factor has also been implicated in antimicrobial resistance. For example, MSMEG_6129 is a putative anti-sigma factor in *M. smegmatis* and deletion of this gene has been shown to confer resistance to chloramphenicol, isoniazid, and tetracycline (Bowman & Ghosh 2014). Consistently, subsequent RNA-Seq analysis showed that the deletion of this gene could lead to the induction of many resistance genes (e.g. genes encoding efflux proteins and transcription regulators), suggesting a possible role of the MSMEG_6129 anti-sigma factor in antibiotic resistance. However, the cognate sigma factor of the MSMEG_6129 anti-sigma factor was not identified in this study.

CHAPTER 3

METHODOLOGY

The overall experimental design of this study is described in Figure 3.1.

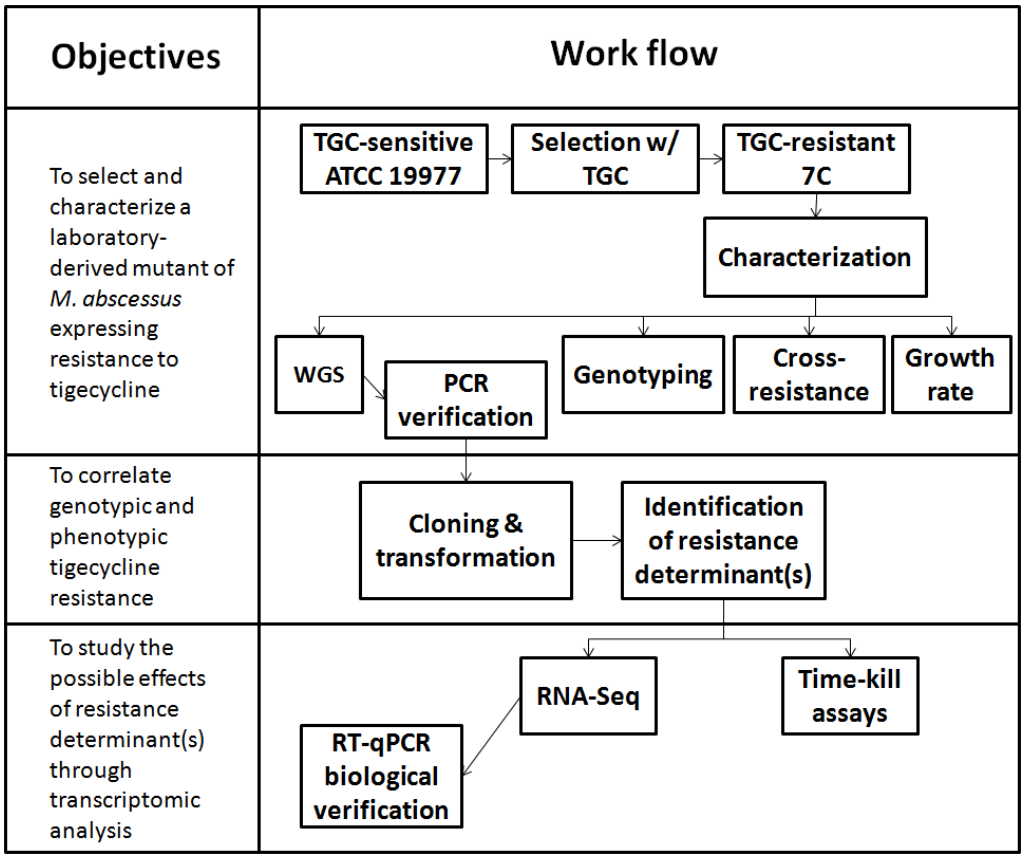

Figure 3.1: Overview of the experimental design of the project. TGC: Tigecycline; WGS: Whole-genome sequencing; RNA-Seq: RNA sequencing.

3.1 Bacterial cultivation and storage

Mab strains were routinely cultured at 37 ℃ on nutrient agar (Becton Dickinson [BD]) or in tryptic soy broth (TSB) (BD). All culture media used in this study were prepared as described in Appendix A. For long-term storage, cultures were frozen at -80 ℃ in broth with 15 % glycerol (Sigma-Aldrich).

3.2 Mutant selection

Spontaneous mutants of *Mab* ATCC 19977 were selected based on the principles of the Luria-Delbrück experiment (Luria & Delbrück 1943). In brief, a small number (~10^3) of *Mab* ATCC 19977 cells was used to inoculate parallel cultures in a non-selective TSB. These parallel cultures were then grown to saturation (~10^8 cells) and plated onto nutrient agar containing tigecycline (Sigma-Aldrich) at a concentration 4×, 8×, 16×, and 32× higher than the MIC of ATCC 19977 (0.25 mg/L). Two to three colony-forming units (CFUs) growing on each agar plate were screened with the Stokes disk diffusion test (BSAC 1991) (see section 3.3.1.2). All CFUs with inhibition zone diameters smaller than that of the control by at least 5 mm were re-tested twice for confirmation. The most resistant mutant, 7C, was selected for futher characterizations. Broth microdilution was used to determine the MIC of tigecycline (see section 3.3.2.2).

3.3 Antimicrobial susceptibility testing

S. aureus ATCC 29213 and ATCC 25923 were included as positive controls for all antibiotic susceptibility testing in this study.

3.3.1 Disk diffusion

3.3.1.1 Kirby-Bauer disk diffusion

Disk diffusion was performed according to the Kirby-Bauer procedure (Bauer et al. 1966). Briefly, an inoculum was prepared by emulsifying growth from solid media in saline (0.9 % sodium chloride [Sigma-Aldrich] in distilled water) and adjusted to 0.5 Mcfarland (~1.5×10^8 CFU/mL). Then, a sterile cotton swab was dipped into the adjusted suspensions. Excess inoculum was removed by rotating the swab while pressing it against the inner wall of the tube above the fluid level. To prepare a bacterial lawn, a pre-dried cation-adjusted Mueller-Hinton agar (CAMHA) (Isolab) was streaked with the swab in one direction. Then, the plate was rotated 90 °and the streaking was repeated using the same swab in that direction. The rotation and streaking were repeated once more. Using a pair of sterile forceps, antibiotic-impregnated discs (BD) were placed onto the plate. For *Mab*, the plate was incubated at the recommended temperature of 30 ℃ for 3-5 days. Using a ruler, inhibition zone diameters were measured in millimeter (mm).

3.3.1.2 Stokes disk diffusion

The susceptibility pattern of a test strain was compared against that of ATCC 19977 (control strain) using Stokes variation of disk diffusion (BSAC 1991). The procedure was identical to the Kirby-Bauer method (see section 3.3.1.1), with the following exceptions: (a) a pre-dried CAMHA plate was divided into two halves, with both test and control strains streaked evenly across each half of the plate, leaving a distance of not more than 5 mm between the two strains; and (b) an antibiotic-impregnated disc was placed at the center of the gap between the test and control strains.

3.3.2 Determination of minimum inhibitory concentration

Etest (Thermo Scientific) or broth microdilution (Woods et al. 2011) was used to determine the MIC, the lowest antibiotic concentration which inhibited visible growth of bacteria. The MICs of tested strains were interpreted according to the values recommended by the CLSI (Woods et al. 2011). As breakpoints for tigecycline have not been defined for mycobacteria, the tigecycline MICs were interpreted according to the non-species related breakpoints set by EUCAST (2018).

3.3.2.1 Etest

Etest was performed according to the manufacturer's protocol. The procedure was identical to the Kirby-Bauer method (see section 3.3.1.1), except for: (a) for *Mab*, inocula were made to match 1 McFarland turbidity standard (b) instead of CAMHA, CAMHA supplemented with 5 % sheep blood (Thermo Scientific) was used; and (c) instead of a disc, an M.I.C. Evaluator strip (Thermo Scientific) was placed onto the bacterial lawn.

3.3.2.2 Broth microdilution

Broth microdilution was performed according to the standard recommended by CLSI (Woods et al. 2011). In brief, a 96-well non-treated microtiter plate (NEST), filled with serial two-fold dilutions of an antibiotic in freshly prepared cation-adjusted Mueller-Hinton broth (CAMHB) (BD), was inoculated with the test organism at a final concentration of 5×10^5 CFU/mL. For *Mab*, the plate was incubated at the recommended temperature of 30 ℃ for 3-5 days. A viable control (the test organism in CAMHB without the antibiotic) was included. Each test organism was tested in duplicate.

3.4 Molecular biology analyses

3.4.1 Nucleic acid preparation

Total DNA was purified from plate cultures using ZR Fungal/Bacterial DNA Miniprep (Zymo Research), following the manufacturer's instructions. The quality and quantity of the DNA samples were assessed using the NanoPhotometer P 300 (Implen) and gel electrophoresis at 0.8 % agarose.

RNA samples were prepared from log phase broth cultures (in CAMHB incubated at 37 ℃ with shaking, at optical density at 600 nm [OD600] of 0.1 to 1). Through rapid agitation using the Vortex Genie 2 (Scientific Industries), bacterial cells were mechanically lysed in the ZR BashingBead Lysis tubes (Zymo Research) with the buffer RLT (part of the RNeasy Mini Kit, Qiagen). RNA samples were purified using the RNeasy Mini Kit with on-column DNase treatment (Qiagen). On-column DNase treatment was insufficient to completely remove the genomic DNA (gDNA) (Appendix B). To minimize the gDNA contamination, DNase digestion in solution was performed on the eluted RNA samples, followed by a cleanup with the RNeasy column which included another round of on-column DNase treatment. The RNA samples were inspected by NanoPhotometer P 300 and the 2100 Bioanalyzer RNA 6000 Nano Assay (Agilent) or gel electrophoresis at 0.8 % agarose.

3.4.2 Next-generation sequencing

3.4.2.1 Whole-genome sequencing

The DNA sample of the mutant 7C was submitted to Beijing Genomics Institute for preparation of a PCR-free library using an undisclosed proprietary method. The constructed library was then sequenced by Illumina HiSeq 4000, using a 2 × 150 bp sequencing protocol. The reads generated were pre-processed with FastX toolkit version 0.0.13.2 (http://hannonlab.cshl.edu/fastx_toolkit/). Clean paired-end reads (with Phred score >20 and free of ambiguous bases) were mapped to the reference genome of ATCC 19977 (National Center for Biotechnology Information [NCBI] Accession: GCF_000069185.1) using bowtie2 version 2.2.1 (Langmead & Salzberg 2012). The average depth (coverage) of reference mapping was 242×. The key metrics of the sequencing, quality filtering and reference mapping are summarized in Table 3.1. The DNA sequence of 7C was uploaded to the GenBank database (accession number: CP030860).

Single nucleotide variants and insertion/deletion mutations were called using SAMtools mpileup version 0.1.19-44428cd with default parameters (Li et al. 2009). Identified variants were then annotated by Annovar (Wang et al. 2010) which utilized the feature file retrieved from the reference genome. Mutations in the coding DNA sequences (CDS) with quality score >50 were considered for subsequent PCR verification.

Table 3.1: Key metrics of the whole-genome sequencing, quality filtering and reference mapping for 7C.

No. of raw reads	Post-filtered reads			Mapping statistics	
	No. of reads	No. of paired reads	No. of orphan reads	No. of reads used	Mapping rate (%)
10,248,578	9,409,374	8,936,264	473,110	8,936,264	97.92

3.4.2.2 RNA-Seq

The RNA samples of 7C and ATCC 19977 with RNA Integrity Number (RIN) >9 (Appendix C) were subjected to rRNA depletion using the Ribo-Zero rRNA Removal Kit (Bacteria) (Illumina). The NEBNext Ultra RNA Library Prep Kit for Illumina (New England Biolabs [NEB]) was used to construct the cDNA libraries. The libraries were sequenced by Illumina HiSeq 4000, using a 2 × 150 bp sequencing protocol. The reads generated were pre-processed with Trimmomatic version 0.38 (Bolger et al. 2014), using the criteria described as follows: (a) bases at both extreme ends of a read with Phred score <20 were removed; (b) if the average Phred score in any sliding window of 8 bp was less than 20, the remaining bases towards the 3' end were removed; (c) reads with length <50 bp at this step was eliminated; and lastly (d) orphan reads after pre-processing were also removed. Clean reads were then mapped to the reference genome of ATCC 19977 (NCBI Accession: GCF_000069185.1) using bowtie2 version 2.2.1 (Langmead & Salzberg 2012). Key metrics of the sequencing, quality filtering and reference mapping are summarized in Table 3.2. The Sequence Read Archive (SRA) accession number for the RNA-Seq sequences of 7C and ATCC 19977 is SRP161545. Read-count normalization and

differential expression analysis were carried out by Cuffdiff (Trapnell et al. 2013). Genes with significant difference in expression between the mutant and its wt counterpart (q-value [adjusted p-value after multiple testing correction] <0.05) were classified as differentially expressed genes (DEGs).

The Database for Annotation Visualization and Integrated Discovery (DAVID) version 6.8 (Huang et al. 2009) was used to study biological functions enriched in the up-regulated (q-value <0.05, fold change ≥1.5) and down-regulated (q-value <0.05, fold change ≤-1.5) DEGs in the mutant. Functions satisfying the Expression Analysis Systematic Explorer (EASE) p-value <0.05 and number of mapped genes >3 were considered as significantly enriched functions.

Table 3.2: Key metrics of the sequencing, quality filtering and reference mapping for 7C and ATCC 19977 for RNA-Seq.

Sample	No. of raw reads	Post-filtered reads			Mapping statistics	
		No. of reads	No. of paired reads	No. of orphan reads	No. of reads used	Mapping rate (%)
7C (1)	8,359,308	8,126,838	7,907,878	218,960	7,907,878	97.60
7C (2)	8,313,438	8,072,888	7,845,630	227,258	7,845,630	98.02
7C (3)	8,341,684	8,139,020	7,948,662	190,358	7,948,662	97.35
ATCC 19977 (1)	8,458,962	8,251,207	8,056,312	194,895	8,056,312	97.47
ATCC 19977 (3)	8,278,454	8,075,769	7,884,426	191,343	7,884,426	98.06
ATCC 19977 (4)	8,197,744	8,023,395	7,858,942	164,453	7,858,942	97.46

3.4.3 End-point PCR

All primers used in this study were synthesized by Integrated DNA Technologies (Table 3.3). Unless otherwise stated, the following conditions were used for all PCR analyses. The PCR cocktail, containing 1 × GoTaq Green Mastermix (Promega), 0.2 μM forward and reverse primers, nuclease-free water, and 10 ng of purified DNA (see section 3.4.1), was prepared. Bacterial CFUs were used as an alternative source of template DNA. Amplification was performed in the Veriti Thermal Cycler (Applied Biosystems [ABI]) using the following profile: 1 cycle of 95 ℃ for 10 minutes, 35 cycles of 95 ℃ for 30 seconds, annealing temperature (Table 3.3) for 30 seconds, and 72 ℃ for 60 seconds, and 1 cycle of 72 ℃ for 10 minutes. A no-template control (NTC) was included in every run. The PCR products were resolved with gel electrophoresis at 2 % agarose. The SYBR Safe (Thermo Scientific)-stained gel was examined under the ultraviolet (UV) in the Biospectrum 410 Gel Documentation System (UVP). When necessary, PCR products were submitted to Apical Scientific Ltd for Sanger sequencing using the BigDye Terminator v3.1 Cycle Sequencing kit and the 3730XL Genetic Analyzer (ABI).

Table 3.3: Primers used in this study.

Primer	Sequence (5'-3')	Target	Estimated size (bp)	Annealing temperature (℃)	Purpose	Reference
TR45-F	CGAACTGCCTC GTGATCG	TR45	Variable	61	Genotyping[1]	Wong et al. 2012
TR45-R	CACTCTCCTGAC GCCAGAC					
TR109-F	GCGTGTGGGCAT ATCAATTA	TR109	Variable	61		
TR109-R	CAATCTCGAGGT GGATGTGA					
TR116-F	GAACACCTCAA CCGCAGTG	TR116	Variable	61		
TR116-R	ATTAGCGCGATA GGCTCACC					
TR150-F	ACGTGGCATCTC GATTGG	TR150	Variable	61		
TR150-R	TCCCACGAGAC CATCAGAAT					
TR155-F	CAACGTGGAAT CTCAATACGC	TR155	Variable	61		
TR155-R	CCCTTGAACAAT TCGAGGAA					
TR172-F	CGTGTAGTCGCT TTGTGCTC	TR172	Variable	61		
TR172-R	ACTAACCATCCC CCACGAC					
1-F	AGCAGGTGATC CGACATGAT	Partial *MAB_0001*	312	60	Verification of whole-genome sequencing findings	This study
1-R	TGGTGAGTGGT TTGACGAGG					
280-F	CGACGCCGAAC CTGATGATT	Partial *MAB_0280*	308	60		
280-R	GTAACAGCACA GCCACAAGG					
748-F	TTCCATCCGCTC TCGGGAA	Partial *MAB_0748*	640	60		
748-R	TTTCACCCATCA CACGAGCC					
1137-F	AGGCTTCACCG ACAAACAGT	Partial *MAB_1137c*	643	60		
1137-R	GAAGCCGATCT GTAGCAGGTT					
1459-F	CGCTGCTTTACG GGCTCTAT	Partial *MAB_1459c*	739	60		
1459-R	ACCATGCCTTCG TATCGGTC					

[1]The primers were designed to amplify variable-number tandem-repeats for genotyping purpose and hence, different strains of *Mycobacterium abscessus* may produce different amplicon sizes. The sequences of the EcoRI recognition site are in bold while the sequences of the HindIII recognition site are underlined. [2]The primers designed to amplify the coding DNA sequence of *MAB_1459c* failed to produce a single specific band, even at an annealing temperature of 80 ℃. Hence, the amplicon of expected size was gel-extracted for cloning.

Table 3.3 (continued): Primers used in this study.

Primer	Sequence (5'-3')	Target	Estimated size (bp)	Annealing temperature (℃)	Purpose	Reference
1587-F	ATGAATTG CGCGACCG TATC	Partial *MAB_1587c*	409	60	Verification of whole-genome sequencing findings	This study
1587-R	CCCAGGTA GTAGGGGT TCCA					
2106-F	ACCGTCTT TACCCTCG ACCT	Partial *MAB_2106c*	590	60		
2106-R	TAGTCGTA GTCGTCAT CGGG					
2537-F	GCGCGAGC ACATAGAG AAGA	Partial *MAB_2537c*	737	60		
2537-R	TCTGTTTG ATGCCTTG TCGGG					
3542-F	TGATCGCG GAAGTGTG GAC	Partial *MAB_3542c*	211	60		
3542-R	ACAGTGGT CTGGCTGA TCTTC					
ORF748-F	TAAG**GAAT TC**ATGACC GCGACTCT CGAC	*MAB_0748*	929	64	Cloning	
ORF748-R	CCGTAAGC TTTCAAAT CTTCTTCG GCGCGA					
ORF1459-F	TAAG**GAAT TC**ATGGCA AACGAGTC GATTCG	*MAB_1459c*	1628	80[2]		
ORF1459-R	CCGTAAGC TTCTAAAC AGGCACCG CGAG					

[1]The primers were designed to amplify variable-number tandem-repeats for genotyping purpose and hence, different strains of *Mycobacterium abscessus* may produce different amplicon sizes. The sequences of the EcoRI recognition site are in bold while the sequences of the HindIII recognition site are underlined. [2]The primers designed to amplify the coding DNA sequence of *MAB_1459c* failed to produce a single specific band, even at an annealing temperature of 80 ℃. Hence, the amplicon of expected size was gel-extracted for cloning.

Table 3.3 (continued): Primers used in this study.

Primer	Sequence (5'-3')	Target	Estimated size (bp)	Annealing temperature (℃)	Purpose	Reference
ORF1587-F	TAAG**GAATTC**ATGACCAGCAACGAAATCACCA	*MAB_1587c*	1232	70	Cloning	This study
ORF1587-R	CCGTAAGCTTTTACGCGTCCACCAGCTC					
ORF3542-F	TAAG**GAATTC**ATGACCGACGGTGAACTCA	*MAB_3542c*	329	66		
ORF3542-R	CCGTAAGCTTCTAGGAGTTCTCGGCCCG					
pMV261-F	CCAGCGTAAGTAGCGGGGTT	Partial pMV261	194	60		
pMV261-R	AGTCTTTCGACTGAGCCTTTCG					
3542screen-F	CTGTTATGTCGCGGTTGCAC	*MAB_3542c*	596	60	Screening for *MAB_3542c* mutations	
3542screen-R	ACCGGCATGAGAGACTGGAT					
rpsJ-F	GCGCAAGATTGTAGAGACGG	Partial *rpsJ*	95	60	RT-qPCR	
rpsJ-R	ACCGGATAACGCAGTACACG					
3543-F	TCAAAGAGGGCACCAACCTC	Partial *MAB_3543c*	201	60		
3543-R	CGTCAGGTAACCCGTCAAGG					

[1]The primers were designed to amplify variable-number tandem-repeats for genotyping purpose and hence, different strains of *Mycobacterium abscessus* may produce different amplicon sizes. The sequences of the EcoRI recognition site are in bold while the sequences of the HindIII recognition site are underlined. [2]The primers designed to amplify the coding DNA sequence of *MAB_1459c* failed to produce a single specific band, even at an annealing temperature of 80 ℃. Hence, the amplicon of expected size was gel-extracted for cloning.

Table 3.3 (continued): Primers used in this study.

Primer	Sequence (5'-3')	Target	Estimated size (bp)	Annealing temperature (℃)	Purpose	Reference
1512-F	GGAGGTTG TCGAGGTT CAGG	Partial *MAB_1512*	194	60	RT-qPCR	This study
1512-R	GATCGCGG GTGAATTT GTCG					
4545-F	AAGGTGCT ACGTGCCA TGAA	Partial *MAB_4545c*	218	60		
4545-R	TCATAGCG GATCACGA CGAC					
4107-F	TTAGTGAT GAGTGCTG GGCG	Partial *MAB_4107c*	162	60		
4107-R	TGGGACGC ATCGGAAT ATGG					

[1]The primers were designed to amplify variable-number tandem-repeats for genotyping purpose and hence, different strains of *Mycobacterium abscessus* may produce different amplicon sizes. The sequences of the EcoRI recognition site are in bold while the sequences of the HindIII recognition site are underlined. [2]The primers designed to amplify the coding DNA sequence of *MAB_1459c* failed to produce a single specific band, even at an annealing temperature of 80 ℃. Hence, the amplicon of expected size was gel-extracted for cloning.

3.4.4 RT-qPCR

cDNA was synthesized by reverse-transcribing 500 ng of an RNA sample using the High Capacity RNA-to-cDNA kit (ABI). Each cDNA sample was diluted 1:10 for subsequent qPCR analysis. The qPCR cocktail, containing 1× ThunderBird Sybr qPCR Mix (Toyobo), 0.2 μM forward and reverse primers (Table 3.3), nuclease-free water, and 1 μL of cDNA, was prepared.

Each sample was tested in duplicate. Amplification was performed in a Rotor-Gene Q Real-time PCR Cycler (Qiagen) using the following thermal cycling profile: 1 cycle of 95 ℃ for 60 seconds, followed by 30 cycles of 95 ℃ for 15 seconds and 60 ℃ for 60 seconds. Among three candidate housekeeping genes tested (*MAB_1512*, *MAB_4107c*, and *MAB_4545*), RefFinder (Xie et al. 2012) identified *MAB_4107c* as the most stably expressed gene (Appendix D) and hence, it was used as the reference gene for normalization. The amplification efficiency was determined using the standard curve generated with serially 10-fold diluted cDNA (the efficiencies of genes tested ranged from 0.9 to 1.1). Target gene expression was quantified relative to *MAB_4107c* with the mathematical model described by Livak and Schmittgen (Livak & Schmittgen 2001). The amplification specificity of the primers (Table 3.3), designed with Primer-BLAST (Ye et al. 2012), was inspected using the melt curve and gel electrophoresis analyses. No amplification was observed in both NTC and no-reverse transcriptase control.

3.5 Cloning and transformation

3.5.1 Molecular cloning of target genes in *Escherichia coli*

The CDS of target genes were PCR-amplified from wt ATCC 19977 DNA, using KAPA HotStart Ready Mix (KAPA Biosystems) and primers described in Table 3.3. Amplification was performed in the Veriti Thermal Cycler using the following profile: 1 cycle of 95 ℃ for 5 minutes, 30 cycles of 98 ℃ for 20 seconds, annealing temperature (Table 3.3) for 15 seconds, and

72 ℃ for 30 seconds, and 1 cycle of 72 ℃ for 5 minutes. These amplicons were then subjected to PCR cleanup by using the QIAquick PCR Purification Kit (Qiagen). For non-specific amplifications, the product of expected size was excised and gel-purified using the QIAquick Gel Extraction Kit (Qiagen). Double digestion of 1 μg of the cleanup amplicons and the pMV261 plasmid (Figure 3.2) (a kind gift from Dr. Therdsak Prammananan, National Center for Genetic Engineering and Biotechnology, Thailand) was performed using EcoRI-HF and HindIII-HF in the presence of the CutSmart buffer (NEB). The unwanted fragments were removed using the QIAquick PCR Purification Kit. The purified PCR fragments were then ligated to the plasmid fragments (100 ng) at 5:1 insert:vector molar ratio, using the T4 DNA ligase (NEB). NEB 5-alpha competent *E. coli* cells were transformed with 5 μL of ligated products through heat shock at 42 ℃ for 30 seconds. After recovery in super optimal broth with catabolite repression (SOC) (NEB), the cells were plated onto Luria-Bertani (LB) agar (First Base) supplemented with 30 mg/L kanamycin (Gold Biotechnology) and incubated at 37 ℃ overnight. To save time, CFUs were screened for recombinant plasmids using colony PCR (see section 3.4.3). The recombinant plasmids were propagated and purified from the LB broth (First Base) cultures (supplemented with 30 mg/L kanamycin) of the PCR-positive clones using the DNA-spin Plasmid Purification Kit (iNtRON Biotechnology). These purified plasmids were verified by Sanger sequencing to confirm that no mutation was introduced into the insert during the cloning and transformation procedures.

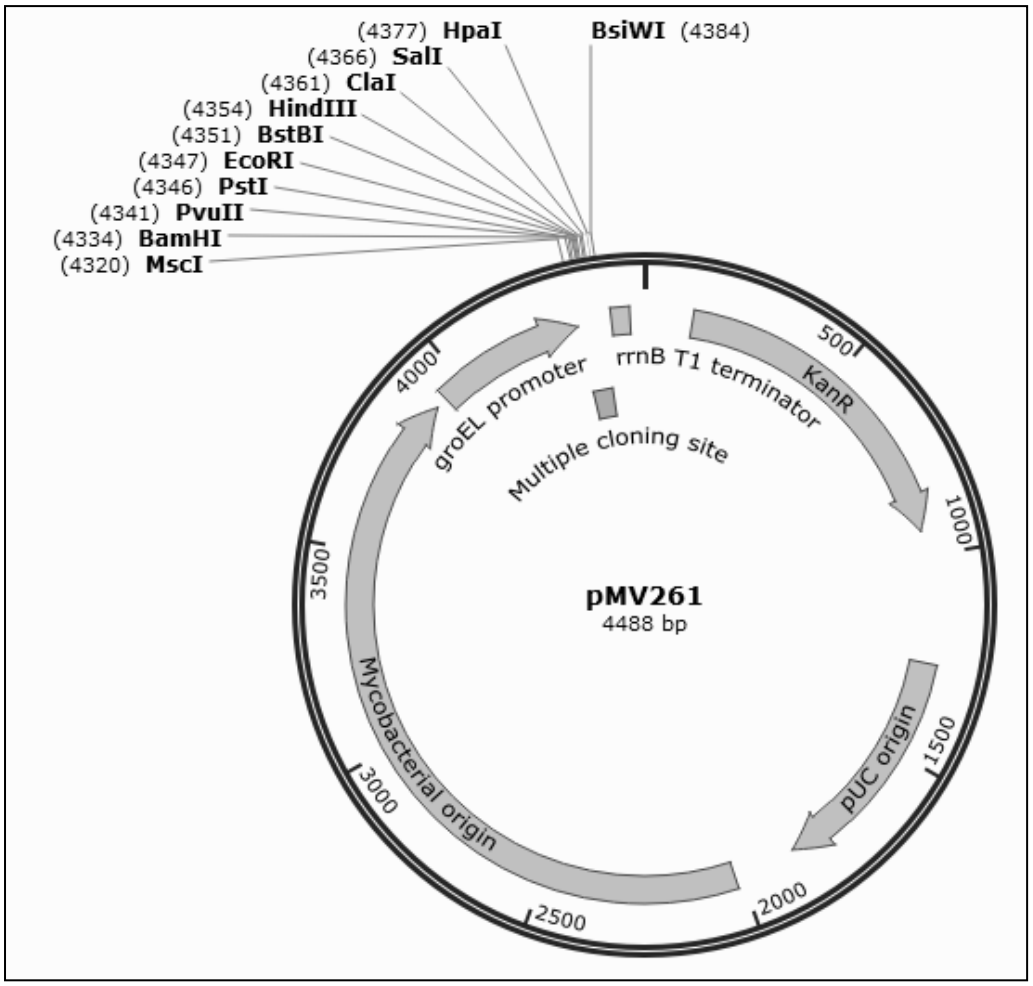

Figure 3.2: Plasmid map of pMV261. This plasmid is a *Mycobacterium-Escherichia coli* shuttle vector which carries the pUC and mycobacterial origins of replication. Target genes were cloned individually into the multiple cloning site, under the control of the constitutive *groEL* promoter. KanR encodes an aminoglycoside phosphotransferase which confers resistance to kanamycin in bacterial hosts. The rrnB T1 terminator is a transcription termination sequence of the *rrnB* gene from *E. coli*.

3.5.2 Transformation of recombinant plasmids into *Mycobacterium abscessus*

3.5.2.1 Preparation of *Mycobacterium abscessus* electro-competent cells

Mab electro-competent cells were prepared as previously described (Broussard 2009). Briefly, log phase *Mab* cells (at OD600 of 0.1 to 1) were harvested from a 100-mL broth culture and washed three times with cold sterile 10 % glycerol. The cells were resuspended in 3 mL of cold 10 % glycerol and were distributed into 100-µL aliquots, which were then frozen on dry ice for 10 minutes and subsequently stored at -80 ℃ for future use.

3.5.2.2 Electroporation of recombinant plasmids into *Mycobacterium abscessus* electro-competent cells

The *Mab* competent cells were mixed with 50 ng of plasmid. The mixture was then transferred into a 0.2-cm electroporation cuvette (Bio-Rad). Electroporation was performed using Eporator (Eppendorf) at 2500 V. After recovery in Middlebrook 7H9 broth (BD), the cells were plated onto Middlebrook 7H10 agar (BD) supplemented with 50 mg/L kanamycin and incubated at 37 ℃ for 3-5 days. Colony PCR was used to screen for plasmid-positive clones.

3.6 Growth/killing-kinetic assays

To set up these assays, log phase broth cultures of *Mab* (at OD600 of 0.1 to 1) were first adjusted to 0.5 McFarland in sterile CAMHB. If necessary, the adjusted cultures were further diluted with CAMHB to a desired concentration (CFU/mL). Unless otherwise stated, broth cultures of growth/killing kinetic assays were shaken at 200 rpm while being incubated at 37 ℃. At different time points, 800 µL of aliquots was transferred into a cuvette (Greiner Bio-One) for OD600 measurement using the NanoPhotometer P 300.

3.6.1 Growth curves in the absence of tigecycline

For each test strain, *Mab* cells were inoculated into 30 mL of CAMHB at a final concentration of 5×10^5 CFU/mL. Aliquots were sampled for quantification every 24 hours for 14 days.

3.6.2 Time-kill kinetics

For each test strain, 6 mL of 0.5-McFarland-adjusted *Mab* cells were exposed to different stress conditions: tigecycline exposure at 8 mg/L and heat exposure at 42 ℃. Cultures were incubated at 37 ℃ (except for those exposed to heat stress) while shaken at the same time. Sampling was performed from each culture at various time points (heat shock: every 4 hours for 12 hours;

tigecycline challenge: every 2 hours for 6 hours).

3.7 BLAST analyses

3.7.1 Identification of orthologs

Protein sequences of *Mab and Mtb* were retrieved from NCBI Gene and Tuberculist (Lew et al. 2011), respectively. Protein Basic Local Alignment Search Tool (BLASTp) (Altschul et al. 1990) was used to identify the corresponding orthologs in the target genome. Only proteins with a BLAST score $\geq$80 and an E-value $\leq 1 \times 10^{-5}$ were considered as potential orthologs.

3.7.2 Identification of gene copy number

The target gene sequence was imported into Nucleotide Basic Local Alignment Search Tool (BLASTn) (Altschul et al. 1990) as the query sequence. The number of matched sequence(s) in the target genome was equivalent to the gene copy number. The *16S rRNA* gene sequences of *M. smegmatis* (Entrez gene ID: 4531789), *Mab* (Entrez gene ID: 5963979), and *Mtb* (Entrez gene ID: 2700429) were used as controls for this analysis (Appendix E). *M. smegmatis* was found to have two copies of *16S rRNA* gene in its genome whereas *Mab* and *Mtb* have one each in their respective genomes, corresponding precisely to the numbers reported previously (Ji et al. 1994; Domenech et al. 1994; Wallace et al. 1996).

3.8 Statistical analysis

All quantitative experiments were performed in biological triplicates. Data were expressed in mean ± one standard deviation. Unless otherwise stated, the difference in the mean between two groups was analyzed using the paired or two-sample t-test in Microsoft Excel 2007.

CHAPTER 4

RESULTS

4.1 Antimicrobial susceptibility patterns of the mutant 7C

In this study, mutants of ATCC 19977 were selected on solid media with increasing concentrations of tigecycline. These mutants were screened using Stokes disk diffusion. Due to budgetary constraint, only the most resistant mutant, 7C, was selected for further characterizations.

For antimicrobial susceptibility testing, quality controls for all tested antibiotics were performed with *S. aureus* ATCC 29213 or ATCC 25923. All quality control results were within the acceptable ranges (Appendix F and G). The MIC of tigecycline for 7C was shown to increase from 0.25 to 2 mg/L (Figure 4.1A and Table 4.1). Hence, in comparison to the wt, 7C was deemed resistant to tigecycline by the non-species related breakpoints (sensitive ≤0.25 mg/L, resistant >0.5 mg/L) set by EUCAST (2018). To look for the development of cross-resistance, 7C was also tested against amikacin, chloramphenicol, ciprofloxacin, clarithromycin, clindamycin, doxycycline, imipenem, linezolid, and tetracycline (Table 4.1). Both 7C and the wt were resistant to chloramphenicol, ciprofloxacin, clindamycin, doxycycline, linezolid, and tetracycline but resistance to imipenem (MIC ≥32 mg/L in 7C vs 8 mg/L in the wt) appeared to have arisen alongside tigecycline resistance in

7C (Figure 4.1B and Figure 4.1C), going by CLSI breakpoints (sensitive ≤4 mg/L, 8 mg/L≤intermediate≤16 mg/L, resistant ≥32 mg/L) for imipenem (Woods et al. 2011).

Table 4.1: Antimicrobial susceptibility testing of *Mycobacterium abscessus* 7C and ATCC 19977.

Antibiotic	Inhibition zone diameter (mm)		MIC (mg/L)	
	7C	ATCC 19977	7C	ATCC 19977
Tigecycline	17.7 ±1.5	38.0 ±1.0	2.00	0.25
Imipenem	No zone	18.3 ±1.5	≥32.00	8.00
Amikacin	17.0	19.0	-	-
Chloramphenicol	No zone	No zone	-	-
Ciprofloxacin	No zone	No zone	-	-
Clarithromycin	32.0	30.0	-	-
Clindamycin	No zone	No zone	-	-
Doxycycline	No zone	No zone	-	-
Linezolid	No zone	No zone	-	-
Tetracycline	No zone	No zone	-	-

The mutant 7C was pre-screened with Stokes disk diffusion and if the zone size was smaller than that of ATCC 19977 by at least 5 mm, the test was repeated two more times and data were expressed as mean ± standard deviation. For those with consistent zone sizes, MIC was determined using Etest or broth microdilution.

4.2 Genotyping of 7C

To demonstrate the clonal relationship between 7C and ATCC 19977, both parent and mutant strains were genotyped using the PCR-based *Mab* variable-number-tandem-repeat analysis (MaVA) (Wong et al. 2012). The copy

number of each recommended locus was determined based on the amplicon size and subsequently confirmed by Sanger sequencing. The results showed identical genotypes for both strains (Figure 4.2 and Table 4.2), signifying that 7C was unlikely an outcome of contamination.

4.3 Fitness cost study

Resistance mutations may impart a fitness cost, as they may affect important biological functions in the cell (Melnyk et al. 2014). In the current study, a growth-kinetics study was performed to compare the growth rates of 7C and ATCC 19977 in the absence of tigecycline (Figure 4.3). By comparing the average time taken to achieve OD600 of 2 (mid-log phase), 7C was found to be replicating 1.2-fold slower than its wt counterpart (p-value <0.01) (Figure 4.3B).

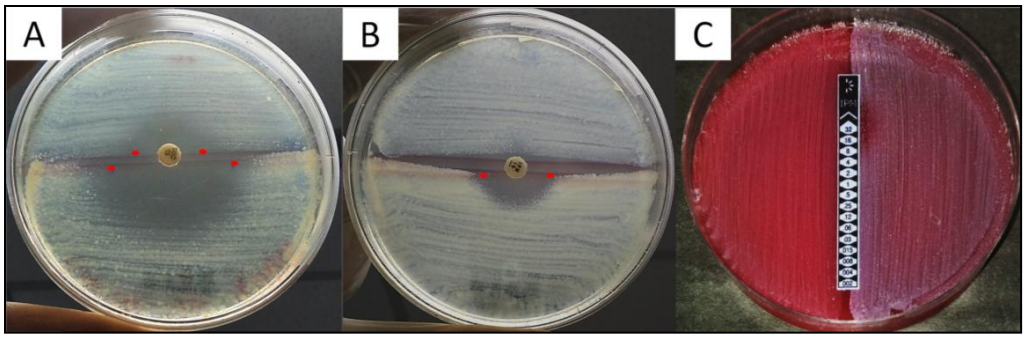

Figure 4.1: Antimicrobial susceptibility testing of 7C and ATCC 19977 using Stokes agar plate disk diffusion and Etest strips. Red dots indicate the inhibition zone size. (A) tigecycline zone sizes of 7C (top) vs ATCC 19977 (bottom); (B) imipenem zone sizes of 7C (top) vs ATCC 19977 (bottom); (C) imipenem Etest of 7C (left) vs ATCC 19977 (right).

Table 4.2: Verification of the *Mycobacterium abscessus* variable-number-tandem-repeat analysis (MaVA) genotyping results by Sanger sequencing.

Locus	Copy number		Expected amplicon size (bp)	Period size (bp)
	7C	ATCC 19977		
TR45	3.4	3.4	206	32
TR109	3.0	3.0	224	32
TR116	4.3	4.3	246	33
TR150	3.6	3.6	227	30
TR155	3.1	3.1	227	31
TR172	4.2	4.2	245	30

Copy numbers of *Mycobacterium abscessus* 7C were calculated from the amplicon sequences using Tandem Repeat Finder. Sequences of ATCC 19977 were retrieved from NCBI. The period size denotes the size of a tandem repeat (TR).

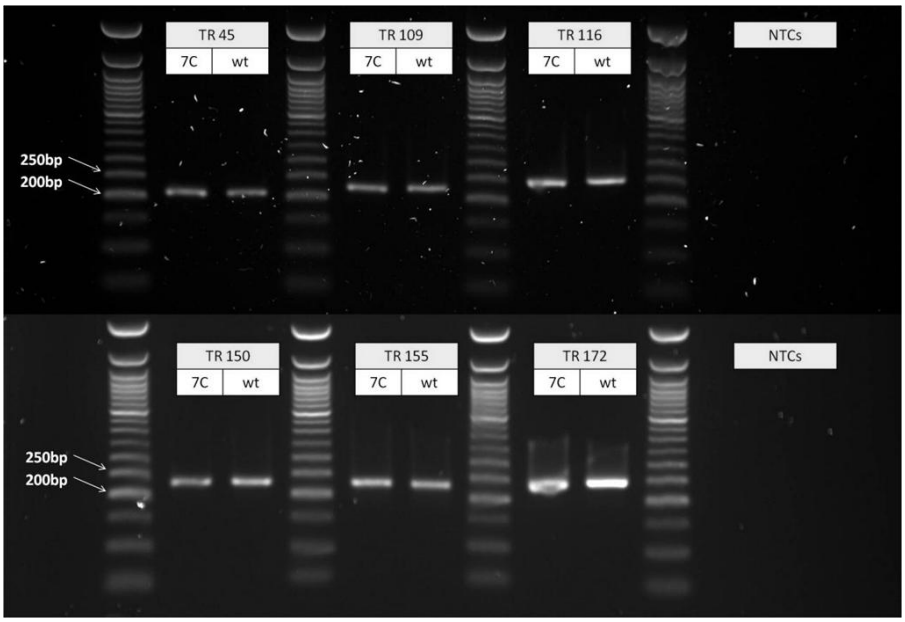

Figure 4.2: *Mycobacterium abscessus* variable-number-tandem-repeat analysis (MaVA) of 7C and wild type (wt) ATCC 19977. The copy numbers of 6 tandem repeat (TR) loci were inferred from the amplicon sizes on gel electrophoresis. The expected amplicon size of each locus is provided in Table 4.2. NTC: No-template control.

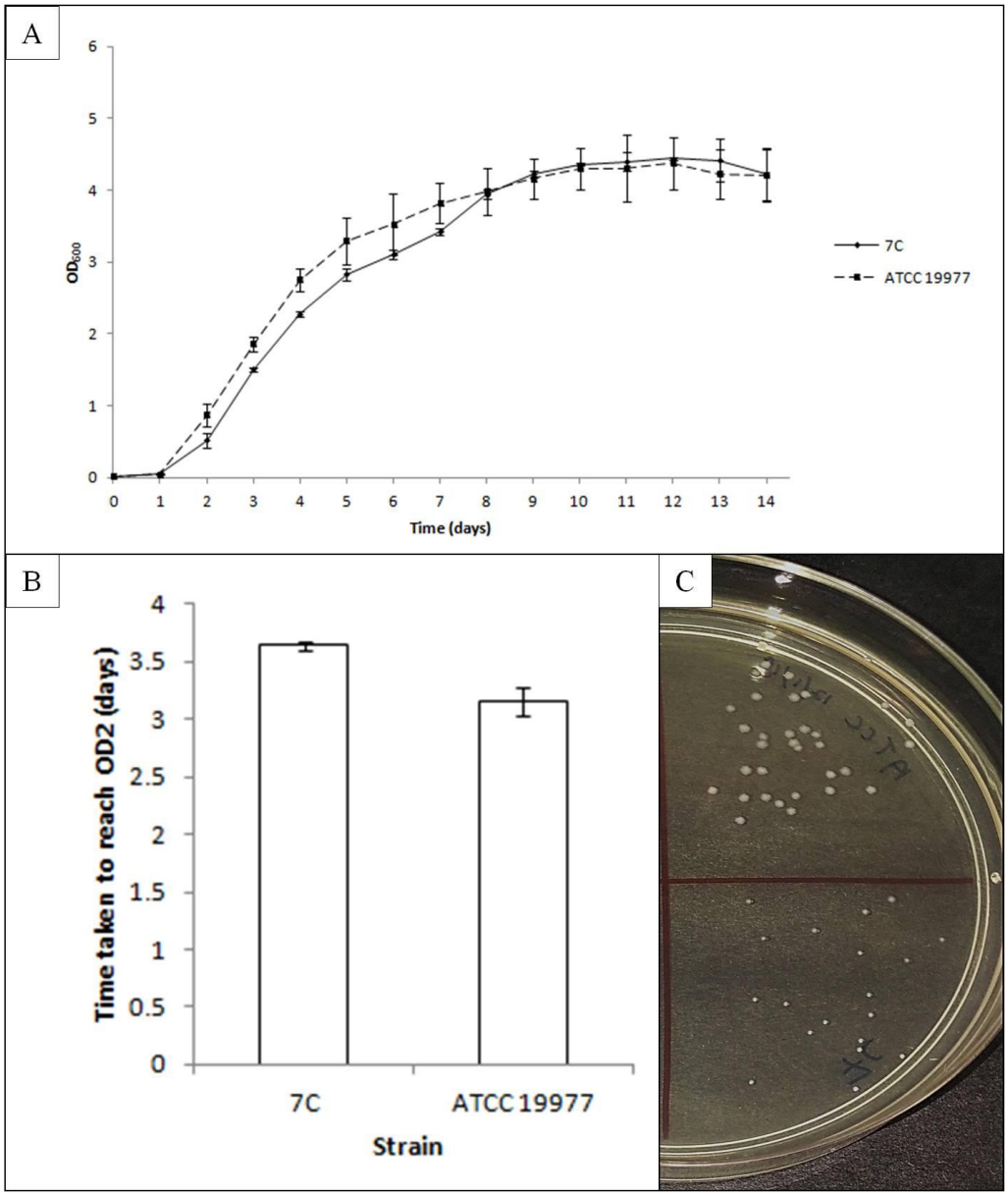

Figure 4.3: Growth rates of *Mycobacterium abscessus* 7C and ATCC 19977 in the absence of tigecycline. (A) Growth kinetics. Data were expressed as mean ± standard deviation of biological triplicates; (B) Average time taken to achieve OD600 of 2; (C) Colony size of 7C (bottom) and ATCC 19977 (top), on solid medium after 4 days of incubation.

4.4 Whole-genome sequencing

To identify the resistance mutation(s), 7C was subjected to WGS. Subsequent variant-calling analysis identified 11 mutations in the CDS with quality score >50 (Table 4.3), which were considered for PCR verification. All 11 mutations were non-synonymous.

Sanger sequencing showed that four of these mutations (*MAB_0280*, *MAB_1137c*, *MAB_2106c*, and *MAB_2537c*) were also found in the laboratory strain of ATCC 19977. Hence, they were unlikely to cause tigecycline resistance in 7C.

In 7C, three insertion mutations were found in *MAB_0001*, the first gene of the *Mab* genome. Interestingly, all of them occurred near the starting point of the gene (Table 4.3). Inconsistently, PCR analysis showed that these mutations were not present in both the mutant and its parental strain. This discrepancy could be due to the errors in the reference assembly (Appendix H). Bacterial genomes are circular in nature. However, during the reference assembly, sequencing reads were mapped to the "linearized" genome. As such, sequencing reads generated from the junction between the first and the last bases of the genome may be misinterpreted as insertions during the variant-calling analysis.

Four mutations were found to be 7C-specific (not found in the laboratory strain of ATCC 19977). These genes were *MAB_0748* 233_234insG,

MAB_1459c G822A, *MAB_1587c* A304G, and *MAB_3542c* T151C. These four candidates were single-copy genes in the ATCC 19977 genome. They were subjected to subsequent biological verification through cloning and transformation.

Table 4.3 Mutations identified by variant-calling analysis (with quality score >50).

Gene	Product	SNP annotation	Mutation	Quality score
MAB_0001	Chromosomal replication initiation protein DnaA	Nonframeshift insertion	5_6insATGCTCGCCGTTGAC	91.7
MAB_0001	Chromosomal replication initiation protein DnaA	Nonframeshift insertion	4_5insGATGCTCGCCGTTGA	79.7
MAB_0001	Chromosomal replication initiation protein DnaA	Frameshift insertion	3_4insATCGCCGTTG	68.0
MAB_0280	ATP-dependent DNA ligase	Stoploss SNV	T919C	222.0
MAB_0748	Phosphate ABC transporter, permease protein PstA	Frameshift insertion	233_234insG	76.5
MAB_1137c	Membrane protein	Nonsynonymous SNV	A2849T	222.0
MAB_1459c	Adenylate/ guanylate cyclase domain-containing protein	Stopgain SNV	G822A	222.0
MAB_1587c	Acyl-CoA desaturase	Nonsynonymous SNV	A304G	222.0
MAB_2106c	Hypothetical protein	Nonsynonymous SNV	C1070T	222.0
MAB_2537c	Thiamine pyrophosphate-requiring protein	Nonsynonymous SNV	C1513T	222.0
MAB_3542c	Conserved hypothetical protein (possible anti-sigma factor)	Nonsynonymous SNV	T151C	222.0

4.5 Cloning and transformation

PCR products of wt *MAB_0748*, *MAB_1459c*, *MAB_1587c*, and *MAB_3542c* were cloned into the pMV261, a *Mycobacterium-E. coli* shuttle vector, to generate the recombinant plasmids pMV261-*MAB_0748*^{wt}, pMV261-

MAB_1459c^{wt}, pMV261-*MAB_1587c*^{wt}, and pMV261-*MAB_3542c*^{wt}, respectively.

A gene dosage study was conducted through the transformation of the recombinant plasmids, one at a time, into the wt ATCC 19977. In the mycobacterial host, the recombinant plasmid would multiply by using the mycobacterial origin of replication (Figure 3.2), effectively increasing the dosage (i.e. copy number) of the target gene. This would lead to over-expression of the target gene upon transcription from the constitutive *groEL* promoter. *Mab* ATCC 19977 carrying the empty pMV261 (ATCC 19977-pMV261) was included as the control. The over-expression of *MAB_0748*^{wt}, *MAB_1459c*^{wt}, *MAB_1587c*^{wt}, and *MAB_3542c*^{wt} in the wt background did not result in any changes in the level of resistance or susceptibility to tigecycline (Table 4.4).

Complementation experiments were then carried out by transforming the recombinant pMV261 plasmids individually into 7C competent cells through electroporation, generating 7C-*MAB_0748*^{wt}, 7C-*MAB_1459c*^{wt}, 7C-*MAB_1587c*^{wt}, and 7C-*MAB_3542c*^{wt}, respectively. The kanamycin-selected, recombinant plasmid-positive clones were then subjected to PCR-Sanger sequencing to confirm the occurrence of complementation in these isolates (Figure 4.4). Using 7C carrying the empty pMV261 (7C-pMV261) as the control, only complementation with *MAB_3542c*^{wt} was found to revert 7C back to the tigecycline-susceptible phenotype (Figure 4.5A and Table 4.5), an observation that was subsequently confirmed with the broth microdilution test

(7C-*MAB_3542c*^{wt}: 0.125 mg/L, 7C-pMV261: 2 mg/L). This observation implied that *MAB_3542c* T151C was the molecular determinant of tigecycline resistance in 7C. In addition, imipenem sensitivity was restored in 7C-*MAB_3542c*^{wt} (Figure 4.5B), which was subsequently verified by using the Etest (7C-*MAB_3542c*^{wt}: 4 mg/L, 7C-pMV261: ≥32 mg/L) (Figure 4.5C). Interestingly, complementation with *MAB_3542c*^{wt} also increased the growth rate of 7C in the absence of tigecycline (1.1 fold, p-value <0.01), to a level which is comparable to that of the wt ATCC 19977 (Figure 4.6). This suggests that the fitness cost exerted by the T151C mutation in *MAB_3542c* had been lifted through complementation.

Table 4.4: Tigecycline susceptibility patterns of *Mycobacterium abscessus* ATCC 19977 transformed with recombinant plasmids using disk diffusion.

Strain	Plasmid	Tigecycline zone diameter (mm)	
		Target	ATCC 19977-pMV261
ATCC 19977-*MAB_0748*^{wt}	pMV261-*MAB_0748*^{wt}	39.0 ± 1.0	39.7 ± 1.2
ATCC 19977-*MAB_1459c*^{wt}	pMV261-*MAB_1459c*^{wt}	40.7 ± 1.2	41.3 ± 1.5
ATCC 19977-*MAB_1587c*^{wt}	pMV261-*MAB_1587c*^{wt}	40.0 ± 1.0	40.0 ± 0.0
ATCC 19977-*MAB_3542c*^{wt}	pMV261-*MAB_3542c*^{wt}	38.7 ± 1.2	38.3 ± 0.6

The experiment was performed in triplicate. ATCC 19977-pMV261 was included as the control. Data were expressed as mean ± standard deviation.

Table 4.5: Tigecycline susceptibility patterns of *Mycobacterium abscessus* 7C complemented with recombinant plasmids carrying the wild-type genes.

Strain	Plasmid	Tigecycline zone diameter (mm)		Tigecycline MIC (mg/L)	
		Target	7C-pMV261	Target	7C-pMV261
7C-*MAB_3542c*^{wt}	pMV261-*MAB_3542c*^{wt}	39.3 ±1.5	19.0 ±1.0	0.125	2.000
7C-*MAB_0748*^{wt}	pMV261-*MAB_0748*^{wt}	20.7 ±3.2	21.7 ±2.3	-	-
7C-*MAB_1459c*^{wt}	pMV261-*MAB_1459c*^{wt}	18.3 ±2.3	19.3 ±1.2	-	-
7C-*MAB_1587c*^{wt}	pMV261-*MAB_1587c*^{wt}	19.3 ±1.5	20.7 ±0.6	-	-

The experiment was performed in triplicate. 7C-pMV261 was included as the control. Data were expressed as mean ± standard deviation.

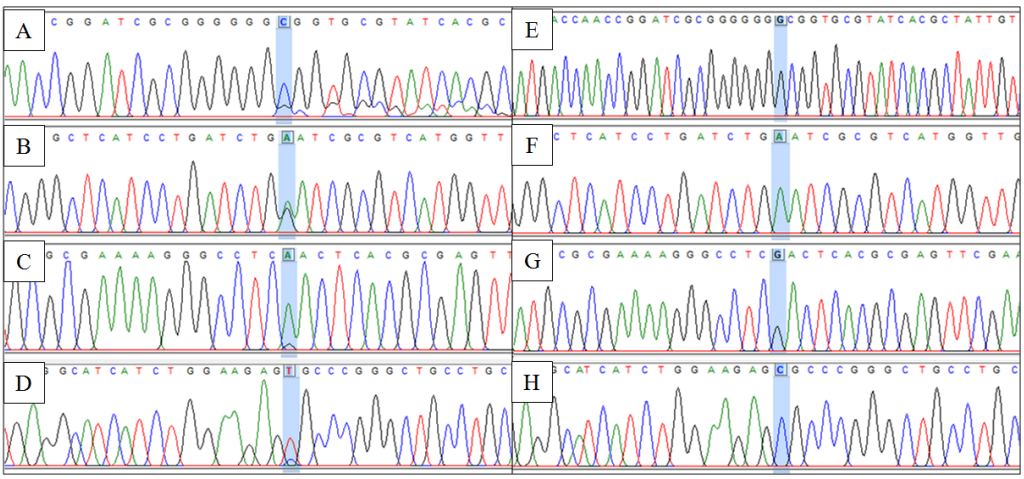

Figure 4.4: Verification of complementation experiments by PCR-Sanger sequencing of target genes. Occurrence of complementation for (A) *MAB_0748* in 7C-*MAB_0748*^{wt}, (B) *MAB_1459c* in 7C-*MAB_1459c*^{wt}, (C) *MAB_1587c* in 7C-*MAB_1587c*^{wt}, and (D) *MAB_3542c* in 7C-*MAB_3542c*^{wt}. Comparing against the gene sequences from 7C-pMV261 ([E] *MAB_0748*, [F] *MAB_1459c*, [G] *MAB_1587c*, and [H] *MAB_3542c*), two expected genotypes (mutated and wild type) were found in every complemented strain. Green peak: A, red peak: T, blue peak: C, and black peak: G.

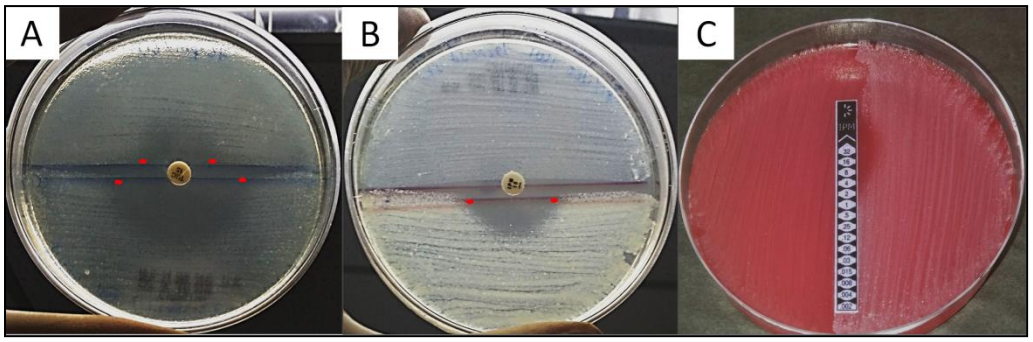

Figure 4.5: Antimicrobial susceptibility testing of 7C-pMV261 and 7C-*MAB_3542c*^{wt} using Stokes agar plate disk diffusion and Etest strips. Red dots indicate the inhibition zone size. (A) tigecycline zone sizes of 7C-pMV261 (top) vs 7C-*MAB_3542c*^{wt} (bottom); (B) imipenem zone sizes of 7C-pMV261 (top) vs 7C-*MAB_3542c*^{wt} (bottom); (C) imipenem Etest of 7C-pMV261 (left) vs 7C-*MAB_3542c*^{wt} (right).

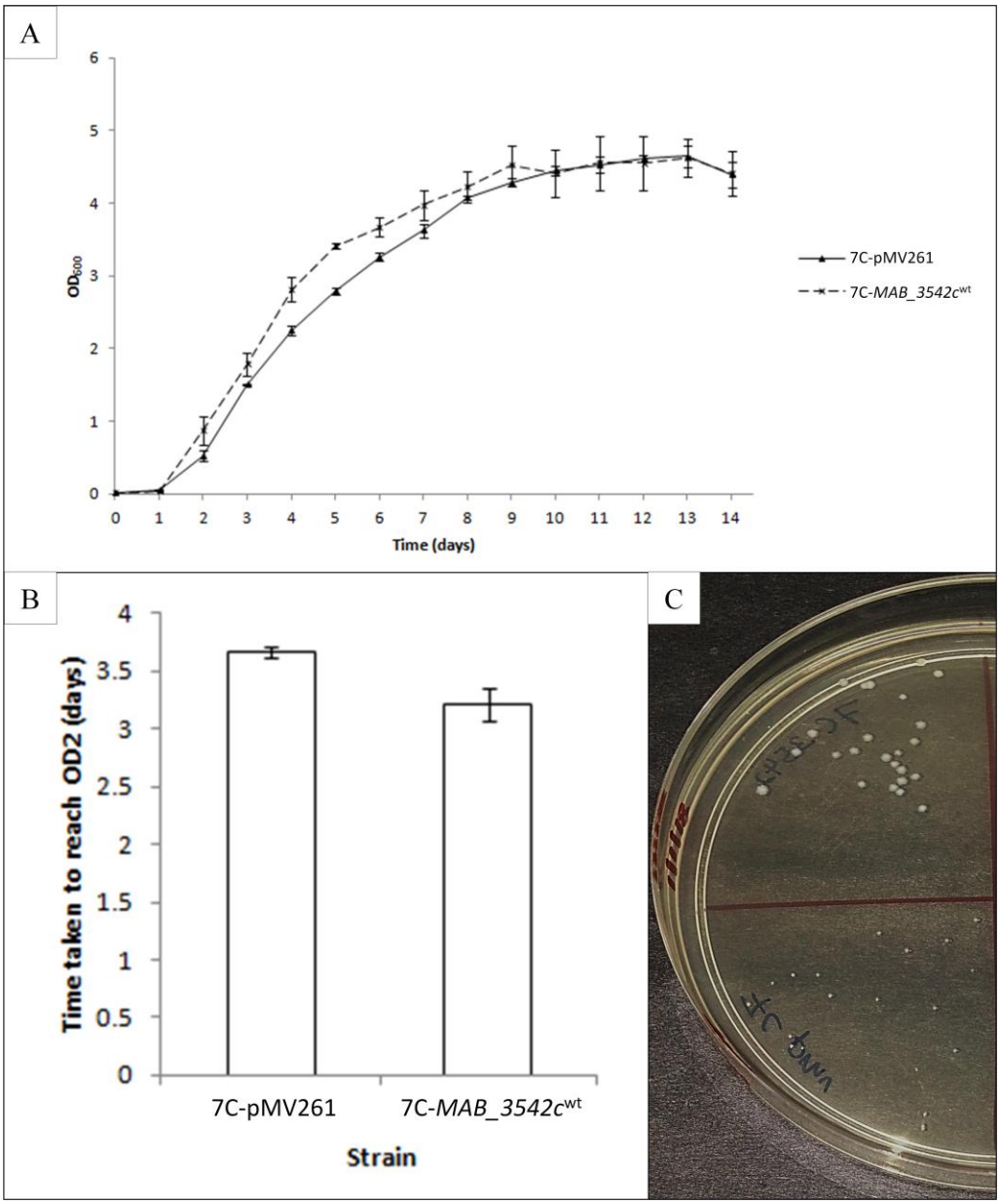

Figure 4.6: Growth rates of *Mycobacterium abscessus* 7C-pMV261 and 7C-*MAB_3542c*^{wt} in the absence of tigecycline. (A) Growth kinetics. Data were expressed as mean ± standard deviation of biological triplicates; (B) Average time taken to achieve OD600 of 2; (C) Colony size of 7C-pMV261 (bottom), and 7C-*MAB_3542c*^{wt} (top) on solid medium after 4 days of incubation.

4.6 Possible role of MAB_3542c

In *Mab*, *MAB_3542c* encodes a 102 amino acid conserved hypothetical protein (possible anti-sigma factor). BLASTp analysis revealed that *Rv3221A* (*rshA*) (BLAST score: 103, E-value: 3×10^{-31}, 75 % query coverage, 70 % identity, 88 % positives [% of identical amino acid + amino acid with similar chemical property]) might be the orthologous gene of *MAB_3542c* in *Mtb* H37Rv (Entrez gene ID: 3205091). Phylogenetic analysis of the MAB_3542c protein along with other anti-sigma factors of *Mtb* H37Rv also suggested that it is related to RshA (Figure 4.7A). Similar to RshA, MAB_3542c also contains a conserved HXXXCXXC motif, of which the two cysteine residues are essential for RshA to negatively regulate the SigH-dependent heat/oxidative stress response (Song et al. 2003). Interestingly, the 7C-specific *MAB_3542c* T151C mutation altered the first cysteine residue in this motif to arginine (Figure 4.7B).

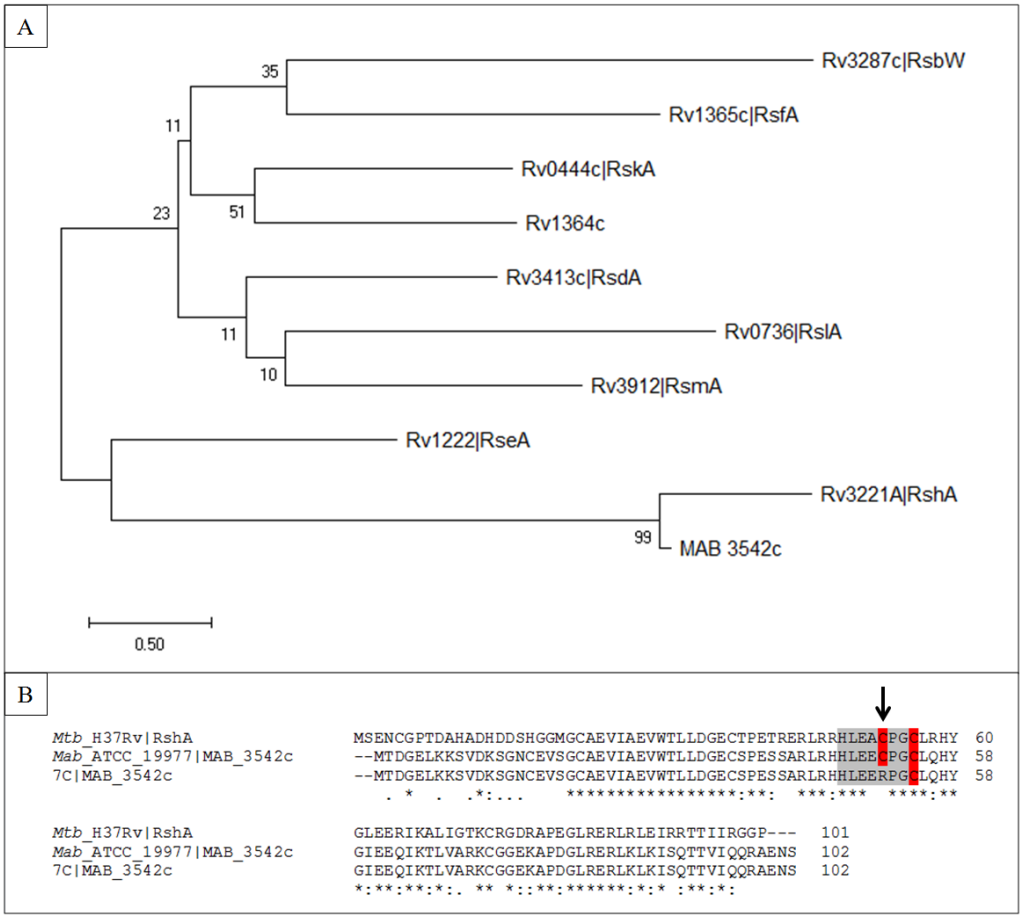

Figure 4.7: MAB_3542c is an RshA-like anti-sigma factor. (A) Phylogenetic analysis of the MAB_3542c protein along with known anti-sigma factors of *M. tuberculosis* H37Rv. The Maximum Likelihood method based on the Whelan and Goldman + Freq. model was used to infer the evolutionary history; (B) multiple sequence alignment of MAB_3542c of *M. abscessus* ATCC 19977 and 7C, and RshA of *M. tuberculosis* H37Rv using Clustal Omega (Sievers et al. 2011). The HXXXCXXC motif is highlighted in grey (the conserved cysteine residues are in red) and the position of the mutation found in 7C is indicated by an arrow. The number at each node indicates a bootstrap value (%). Evolutionary analyses were conducted in MEGA version X (Kumar et al. 2018).

4.7 Screening of MAB_3542c mutations in clinical isolates

To determine the relevance of *MAB_3542c* in mycobacterial clinical isolates, target amino acid sequence variability was compared among 48 patient isolates comprising *Mab* (15 isolates), *M. massiliense* (32 isolates), and *M. bolletii* (1 isolate). The subspecies classification of these isolates was carried out in the previous studies (Wong et al. 2012; Tan et al. 2013). With *Mab* ATCC 19977 as the control, Stokes disk diffusion was used to determine tigecycline susceptibility patterns among these isolates. Of the 48 isolates tested, 22 had inhibition zone diameters at least 5 mm smaller than that of ATCC 19977; three had diameters smaller than that of 7C (Table 4.6). The level of susceptibility was expressed as ratio of zone size relative to that of ATCC 19977. Interestingly, the *M. massiliense* strains were found to be more resistant to tigecycline than the *Mab* strains (p-value <0.01). *M. bolletti* was excluded from this comparison due to the small number of strains tested in this study ($n = 1$).

All 48 isolates were subjected to Sanger sequencing for mutations in the *MAB_3542c* gene. Unexpectedly, irrespective of their susceptibility patterns, no mutation was detected in the amino acid sequences of these clinical isolates, suggesting that the MAB_3542c mutation may not be prevalent among clinical strains. Nonetheless, synonymous mutations (by comparing against ATCC 19977) were found in both *M. massiliense* and *M. bolletii* isolates. The single gene-based tree inferred using *MAB_3542c* was constructed with MEGA version X (Kumar et al. 2018). Interestingly,

MAB_3542c phylogenetic grouping in Figure 4.8 corresponded exactly to the subspecies grouping from previous studies (Wong et al. 2012; Tan et al. 2013), demonstrating that these synonymous mutations may be subspecies-specific and hence, *MAB_3542c* may be useful for the identification of *Mab* subspecies.

Table 4.6: Tigecycline susceptibility patterns of clinical isolates of *Mycobacterium abscessus* complex and 7C.

Strain	Subspecies[1]	Zone size (mm)		Standard deviation		Ratio (Target/ ATCC 19977)
		Target	ATCC 19977	Target	ATCC 19977	
M143	m	11.3	31.7	1.5	1.2	0.36
M162	m	11.7	32.3	1.2	0.6	0.36
M172	m	14.0	34.7	0.0	2.1	0.40
7C	a	17.7	38.0	1.5	1.0	0.47
M215	m	16.3	33.3	3.5	1.2	0.49
M209	m	17.0	33.7	2.6	1.2	0.50
M57	m	16.0	31.3	1.7	2.3	0.51
M206	m	17.0	31.0	2.0	1.0	0.55
M27	m	18.7	33.0	3.8	3.6	0.57
M148	m	20.7	33	0.6	3.0	0.63
M154	m	22.0	34.3	3.0	1.5	0.64
M139	m	24.7	34.7	2.5	0.6	0.71
M33	m	23.7	32.7	3.5	1.2	0.72
M161	m	25.0	34.3	1.0	3.1	0.73
M214	a	25.7	35.0	2.1	2.6	0.73
M145	m	24.0	32.7	1.7	1.5	0.73
M115	m	27.0	35.7	1.0	1.2	0.76

Stokes disk diffusion was used with *M. abscessus* ATCC 19977 as control. All isolates were pre-screened and if the zone size was smaller than that of ATCC 19977 by at least 5 mm, the test was repeated two more times and data were expressed as mean ± standard deviation. The level of susceptibility was expressed as ratio of zone size relative to that of ATCC 19977. [1]Subspecies included a: *M. abscessus*, m: *M. massiliense*, and b: *M. bolletii*. N/A: Not applicable.

Table 4.6 (continued): Tigecycline susceptibility patterns of clinical isolates of *Mycobacterium abscessus* complex and 7C.

Strain	Subspecies[1]	Zone size (mm)		Standard deviation		Ratio (Target/ ATCC 19977)
		Target	ATCC 19977	Target	ATCC 19977	
M152	a	26.7	35.0	2.1	1.0	0.76
M04	m	25.0	32.7	1.0	0.6	0.77
M211	m	25.3	32.3	0.6	1.5	0.78
M02	m	25.3	32.0	1.5	1.0	0.79
M119	m	28.3	35.7	1.2	2.1	0.79
M159	m	28.0	34.0	0.0	2.6	0.82
M134	m	30.0	34.0	N/A	N/A	0.88
B0	m	30.0	33.0	N/A	N/A	0.91
A5	a	31.0	34.0	N/A	N/A	0.91
A2	a	37.0	40.0	N/A	N/A	0.93
M94	a	36.0	38.0	N/A	N/A	0.95
A9	a	31.0	32.0	N/A	N/A	0.97
M117	m	31.0	32.0	N/A	N/A	0.97
M120	m	32.0	33.0	N/A	N/A	0.97
B6	a	37.0	38.0	N/A	N/A	0.97
M149	a	38.0	38.0	N/A	N/A	1.00
M61	a	35.0	35.0	N/A	N/A	1.00
M93	a	35.0	35.0	N/A	N/A	1.00
B14	m	33.0	33.0	N/A	N/A	1.00
M43	m	34.0	34.0	N/A	N/A	1.00
M24	b	34.0	34.0	N/A	N/A	1.00
B12	m	33.0	33.0	N/A	N/A	1.00
M18	m	36.0	36.0	N/A	N/A	1.00

Stokes disk diffusion was used with *M. abscessus* ATCC 19977 as control. All isolates were pre-screened and if the zone size was smaller than that of ATCC 19977 by at least 5 mm, the test was repeated two more times and data were expressed as mean ± standard deviation. The level of susceptibility was expressed as ratio of zone size relative to that of ATCC 19977. [1]Subspecies included a: *M. abscessus*, m: *M. massiliense*, and b: *M. bolletii*. N/A: Not applicable.

Table 4.6 (continued): Tigecycline susceptibility patterns of clinical isolates of *Mycobacterium abscessus* complex and 7C.

Strain	Subspecies[1]	Zone size (mm)		Standard deviation		Ratio (Target/ ATCC 19977)
		Target	ATCC 19977	Target	ATCC 19977	
M156	m	30.0	30.0	N/A	N/A	1.00
M202	m	36.0	35.0	N/A	N/A	1.03
A6	a	37.0	35.0	N/A	N/A	1.06
M237	a	36.0	34.0	N/A	N/A	1.06
M213	m	35.0	33.0	N/A	N/A	1.06
A10	a	41.0	38.0	N/A	N/A	1.08
M127	a	40.0	37.0	N/A	N/A	1.08
A4	a	34.0	31.0	N/A	N/A	1.10
M97	m	41.0	34.0	N/A	N/A	1.21

Stokes disk diffusion was used with *M. abscessus* ATCC 19977 as control. All isolates were pre-screened and if the zone size was smaller than that of ATCC 19977 by at least 5 mm, the test was repeated two more times and data were expressed as mean ± standard deviation. The level of susceptibility was expressed as ratio of zone size relative to that of ATCC 19977. [1]Subspecies included a: *M. abscessus*, m: *M. massiliense*, and b: *M. bolletii*. N/A: Not applicable.

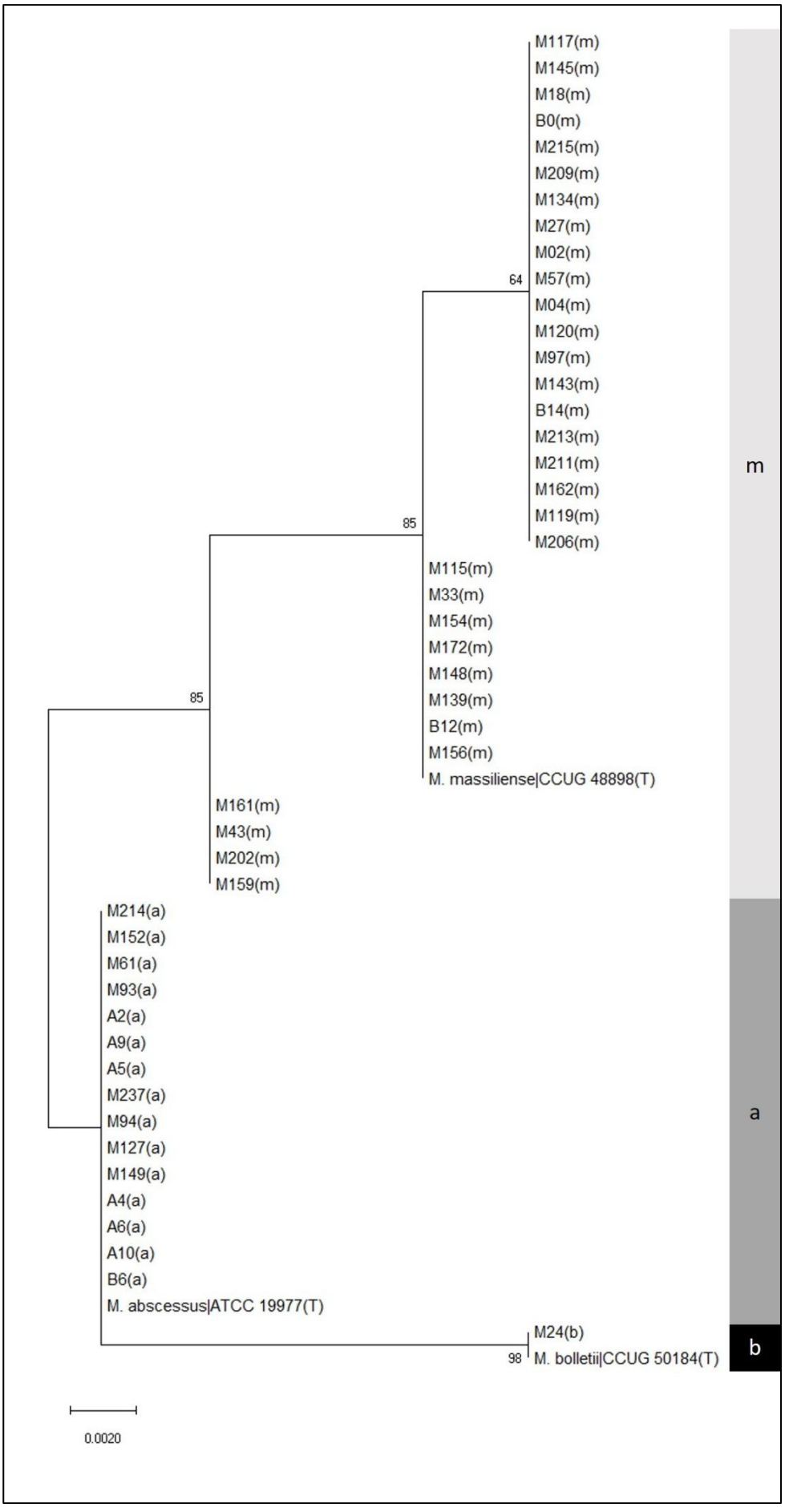
M117(m)
M145(m)
M18(m)
B0(m)
M215(m)
M209(m)
M134(m)
M27(m)
M02(m)
64
M57(m)
M04(m)
M120(m)
M97(m)
M143(m)
B14(m)
M213(m)
M211(m)
M162(m)
M119(m)
M206(m)
85
M115(m)
M33(m)
M154(m)
M172(m)
M148(m)
M139(m)
B12(m)
M156(m)
M. massiliense|CCUG 48898(T)
85
M161(m)
M43(m)
M202(m)
M159(m)
M214(a)
M152(a)
M61(a)
M93(a)
A2(a)
A9(a)
A5(a)
M237(a)
M94(a)
M127(a)
M149(a)
A4(a)
A6(a)
A10(a)
B6(a)
M. abscessus|ATCC 19977(T)
M24(b)
98
M. bolletii|CCUG 50184(T)
0.0020
m
a
b

Figure 4.8: *MAB_3542c* (309 bp)-based phylogenetic tree for the clinical isolates of *Mycobacterium abscessus* complex, which can be divided into three related subspecies: *M. abscessus* (a), *M. massiliense* (m), and *M. bolletii* (b). Based on the Kimura-2 parameter model, the Maximum Likelihood method was used to predict the evolutionary history. The bootstrap consensus tree was inferred from 1000 replicates. The number at each node indicates a bootstrap value (%). Sequences of type strains (T) for each subspecies were retrieved from NCBI. The subspecies classification of clinical isolates (as indicated in the parentheses after the strain name) was carried out in previous studies (Wong et al. 2012; Tan et al. 2013). Evolutionary analyses were conducted in MEGA version X (Kumar et al. 2018).

4.8 Heat-stress response of 7C

Previous studies have shown that heat exposure can lead to the formation of oxygen radicals and trigger the oxidative stress response (Benov & Fridovich 1995; Lüders et al. 2009; Zhang et al. 2017). Since RshA is able to detect both heat and oxidative stresses (Song et al. 2003), the heat stress response of mutant 7C was selectively investigated in this study. As expected, the wt showed significantly less survival than the mutant following exposure to heat at 42 ℃ after 4 and 8 hours, respectively (Figure 4.9A). To determine whether this phenotype was the direct outcome of MAB_3542c mutation, the heat-shock experiment was repeated with 7C-*MAB_3542c*^{wt} and 7C-pMV261. In comparison to the strain of 7C carrying the empty plasmid (7C-pMV261), the complemented strain 7C-*MAB_3542c*^{wt} was more susceptible to heat shock (Figure 4.9B), indicating that the mutation responsible for tigecycline resistance may also be linked to the enhanced heat stress response in 7C.

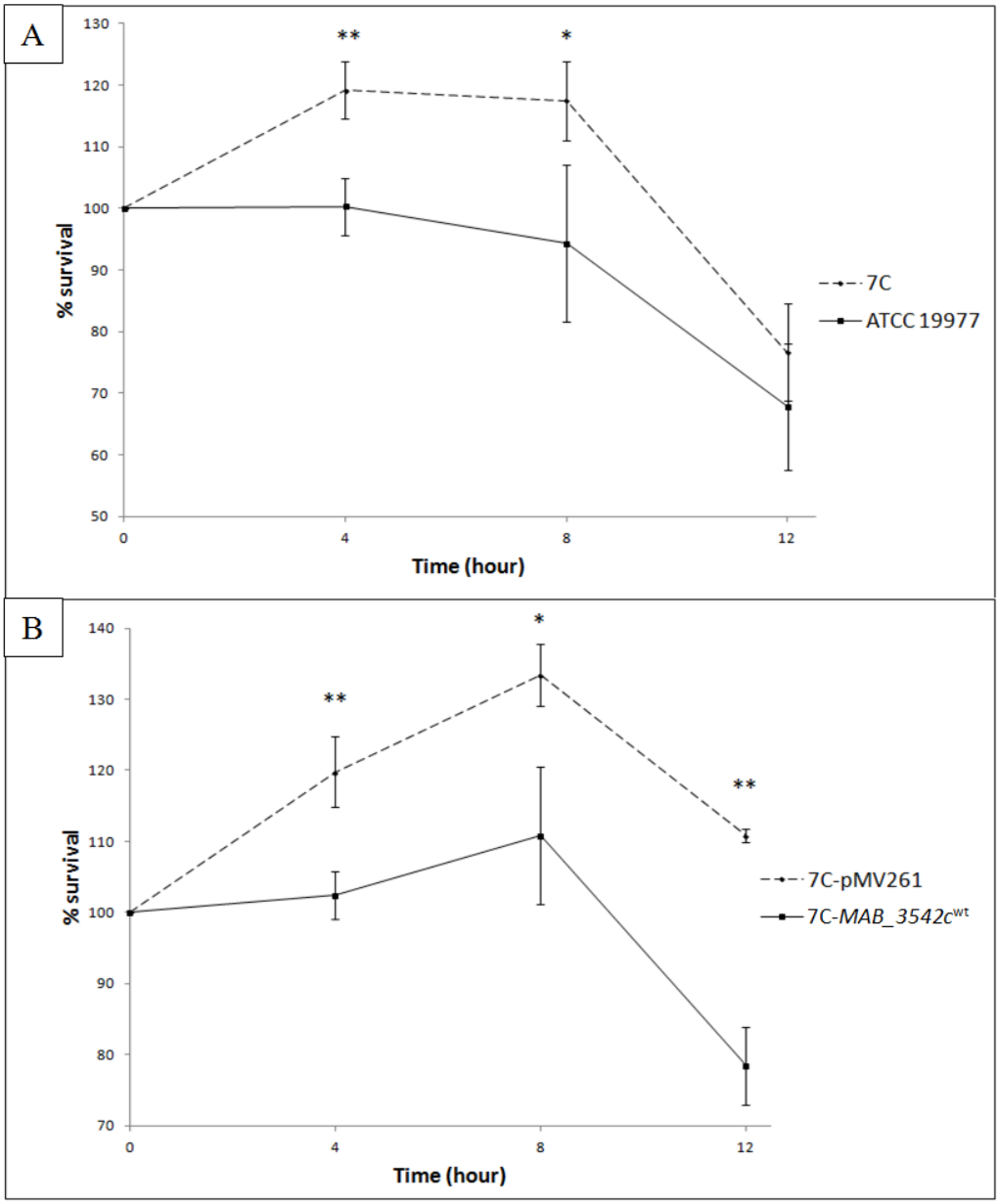

Figure 4.9: Heat-stress response of (A) 7C versus ATCC 19977 and (B) 7C-pMV261 versus 7C-*MAB_3542c*^{wt} at 42 ℃. Data were expressed as mean ± standard deviation of biological triplicates. *p-value <0.05; **p-value <0.01.

4.9 Transcriptomic profiling using RNA-Seq

To investigate the transcriptomic changes elicited by the defect in MAB_3542c, 7C and ATCC 19977 were subjected to RNA-Seq profiling in

biological triplicates. Among 4992 genes in the genome of *Mab*, significant difference in gene expression between the mutant and its wt counterpart (q-value <0.05) was observed in 988 genes (Appendix I and J), of which 491 were differentially expressed by at least 1.5 fold in 7C. Among these DEGs, the changes in their expression were skewed towards up-regulation in the mutant (300 up and 191 down).

The foundation of a functional annotation analysis is that if a biological function is altered in a study, the genes involved in this function should be more likely to be picked up (or enriched) as a relevant group by high-throughput screening technologies. In this study, 300 up-regulated genes (q-value <0.05, fold change ≥1.5) and 191 down-regulated genes (q-value <0.05, fold change ≤-1.5) were used for DAVID functional annotation (Huang et al. 2009), respectively.

4.9.1 Up-regulated genes

Various functions were enriched in the over-expressed genes (Table 4.7). One of them was sigma factor activity, of which *MAB_3543c*, *MAB_1362*, *MAB_3028*, and *MAB_3428c* were among the over-expressed genes of 7C that mapped to this function (Table 4.7).

Heat shock proteins can function as chaperones, which are charged with degrading misfolded or abnormal proteins during heat stress (Craig et al. 1993).

In this study, the chaperone was the most over-represented function in the up-regulated genes of 7C, including *MAB_0650 (groEL), MAB_3731c (groEL protein 1), MAB_3732c (groES), MAB_4265c (clpB),* and *MAB_4271c* (*dnaJ*) (Table 4.7).

Moreover, genes encoding oxidoreductases, such as thioredoxin (*MAB_2739c* and *MAB_4940*), catalase (*MAB_2530c*), peroxidase (*MAB_2270c* and *MAB_0693*), luciferase/luciferase-like protein (*MAB_1339, MAB_3639, MAB_0947c*, and *MAB_4233c*), etc, were found to be up-regulated in 7C. Interestingly, the 13 genes mapped to monooxygenase were a subset of genes mapped to oxidoreductase, suggesting that the former function is a sub-term of the latter function.

Based on previous publications (Raman et al. 2001; Mangan et al. 2002; Manganelli et al. 2002; Song et al. 2003), a list of known *Mtb* genes that are directly under the regulation of SigH was compiled (Figure 4.9 and Table 4.8). Next, BLASTp was used to identify their respective orthologs in *Mab* (Table 4.8). Remarkably, with a lone exception of *MAB_4272c*, all genes mapped to this pathway were significantly up-regulated in 7C, including the orthologous gene of SigH, *MAB_3543c* (Figure 4.10). On the other hand, genes encoding orthologs of the transcriptional repressors HrcA (*MAB_1665*) and HspR (*MAB_4270c*) were not differentially expressed.

Although beta-lactamase activity was not picked up by DAVID analysis as an enriched function, it is worth noting that several beta-lactamase/beta-lactamase-like protein-encoding genes, *MAB_4231* (q-value <0.01, fold change

= 1.3), *MAB_4947* (q-value = 0.01, fold change = 1.3), and *MAB_2179* (q-value <0.01, fold change = 1.6), were up-regulated in 7C. This may explain the resistance phenotype of 7C against imipenem (Figure 4.1B and Figure 4.1C).

Table 4.7: Over-represented functions (p-value <0.05) in up-regulated genes of *Mycobacterium abscessus* 7C (q-value <0.05, fold change ≥1.5).

Gene	Gene product	q-value	Fold change
Chaperone (fold enrichment = 5.8)			
MAB_4265c	Chaperone ClpB	<0.01	2.2
MAB_3732c	10 kDa chaperonin (GroES)	<0.01	1.5
MAB_0650	60 kDa chaperonin 2 (Protein Cpn60 2) (GroEL)	<0.01	1.5
MAB_4271c	Chaperone protein DnaJ	<0.01	1.5
MAB_3731c	60 kDa chaperonin 1 (GroEL protein 1)	<0.01	1.6
Sigma factor (fold enrichment = 5.0)			
MAB_3428c	Probable RNA polymerase sigma-C factor	<0.01	1.5
MAB_3028	RNA polymerase sigma factor	<0.01	3.0
MAB_3543c	RNA polymerase sigma-E factor	<0.01	4.5
MAB_1362	Probable alternative RNA polymerase sigma factor	<0.01	9.2
Monooxygenase (fold enrichment = 3.0)			
MAB_1426	Putative cytochrome P450	<0.01	1.5
MAB_3598c	Putative alkane-1-monooxygenase AlkB (fatty acid omega-hydroxylase)	<0.01	1.6
MAB_2249	Probable lysine-N-oxygenase MbtG	<0.01	1.7
MAB_4456	Putative cytochrome P450	<0.01	1.7
MAB_4050c	Probable monooxygenase	<0.01	1.6
MAB_1601c	Putative monooxygenase	<0.01	1.8
MAB_1339	Putative monooxygenase (luciferase-like)	<0.01	12.4
MAB_3401	Putative monooxygenase	<0.01	2.4
MAB_4232c	Putative oxygenase	<0.01	4.5
MAB_1527	Probable monooxygenase	<0.01	4.4
MAB_4233c	Putative monooxygenase (luciferase-like)	<0.01	5.0
MAB_1324	Putative monooxygenase	<0.05	1.8
MAB_0370	Hypothetical protein	<0.01	1.9

Functions were ranked according to the fold enrichment.

Table 4.7 (continued): Over-represented functions (p-value <0.05) in up-regulated genes of *Mycobacterium abscessus* 7C (q-value <0.05, fold change ≥1.5).

Gene	Gene product	q-value	Fold change
Oxidoreductase (fold enrichment = 1.6)			
MAB_2134	NADH-quinone oxidoreductase, A subunit NuoA	<0.01	1.7
MAB_2446c	Putative pirin-like protein	<0.01	1.8
MAB_2530c	Catalase CatB	<0.01	1.9
MAB_2761	Glucose-6-phosphate 1-dehydrogenase	<0.01	1.6
MAB_3016c	Hypothetical protein	<0.01	27.0
MAB_2270c	Putative peroxidase	<0.01	2.2
MAB_3014	Putative FAD-dependent pyridine nucleotide-disulphide oxidoreductase, similar to mercuric reductases protein	<0.01	1.6
MAB_3598c	Putative alkane-1-monooxygenase AlkB (fatty acid omega-hydroxylase)	<0.01	1.6
MAB_0830	Probable NADH-dependent flavin oxidoreductase	<0.01	5.1
MAB_4456	Putative cytochrome P450	<0.01	1.7
MAB_2249	Probable lysine-N-oxygenase MbtG	<0.01	1.7
MAB_4162c	Putative 2-nitropropane dioxygenase	<0.01	1.6
MAB_0120	Probable peptide methionine sulfoxide reductase	<0.01	1.7
MAB_4050c	Probable monooxygenase	<0.01	1.6
MAB_1601c	Putative monooxygenase	<0.01	1.8
MAB_2740c	Probable oxidoreductase	<0.01	3.5
MAB_3377	Hypothetical protein	<0.01	13.2
MAB_1339	Putative monooxygenase (luciferase-like)	<0.01	12.4
MAB_2739c	Probable thioredoxin TrxB	<0.01	12.6
MAB_1527	Probable monooxygenase	<0.01	4.4
MAB_2595	Putative pyridoxamine 5'-phosphate oxidase	<0.01	2.7
MAB_1324	Putative monooxygenase	<0.05	1.8

Functions were ranked according to the fold enrichment.

Table 4.7 (continued): Over-represented functions (p-value <0.05) in up-regulated genes of *Mycobacterium abscessus* 7C (q-value <0.05, fold change ≥1.5).

Gene	Gene product	q-value	Fold change
Oxidoreductase (fold enrichment = 1.6) (continued)			
MAB_0370	Hypothetical protein	<0.01	1.9
MAB_3639	Putative luciferase-like protein	<0.01	8.8
MAB_1426	Putative cytochrome P450	<0.01	1.5
MAB_4434c	Putative iron/ascorbate dependent oxidoreductase	0.04	1.5
MAB_0485	Putative oxidoreductase	<0.01	3.7
MAB_4295c	Probable UDP-glucose 6-dehydrogenase (UdgA)	<0.01	9.4
MAB_4735	Putative starvation-induced DNA protecting protein/Ferritin and Dps	<0.01	11.4
MAB_2140	NADH-quinone oxidoreductase, G subunit NuoG	<0.01	1.8
MAB_2244	Fumarate reductase/succinate dehydrogenase flavoprotein-like FrdA	0.04	1.6
MAB_3487	Probable acyl-CoA dehydrogenase	<0.01	1.5
MAB_3486	Probable acyl-CoA dehydrogenase	<0.01	2.0
MAB_0659	Putative dioxygenase	<0.01	2.4
MAB_4940	Thioredoxin reductase (TrxB)	<0.01	2.3
MAB_0827	Hypothetical short-chain dehydrogenase/reductase	<0.01	6.6
MAB_0693	Probable glutathione peroxidase	<0.01	1.7
MAB_3401	Putative monooxygenase	<0.01	2.4
MAB_4232c	Putative oxygenase	<0.01	4.5
MAB_3400	NADP-dependent alcohol dehydrogenase C	<0.01	2.2
MAB_3900c	Hypothetical protein	<0.05	2.5
MAB_0947c	Putative luciferase	<0.01	1.7
MAB_4234c	Putative FMNH2-utilizing oxygenase	<0.01	15.4
MAB_4233c	Putative monooxygenase (luciferase-like)	<0.01	5.0
MAB_4437	Probable acyl-CoA dehydrogenase FadE	<0.01	2.5
MAB_2047c	Probable ferredoxin reductase	<0.01	1.5

Functions were ranked according to the fold enrichment.

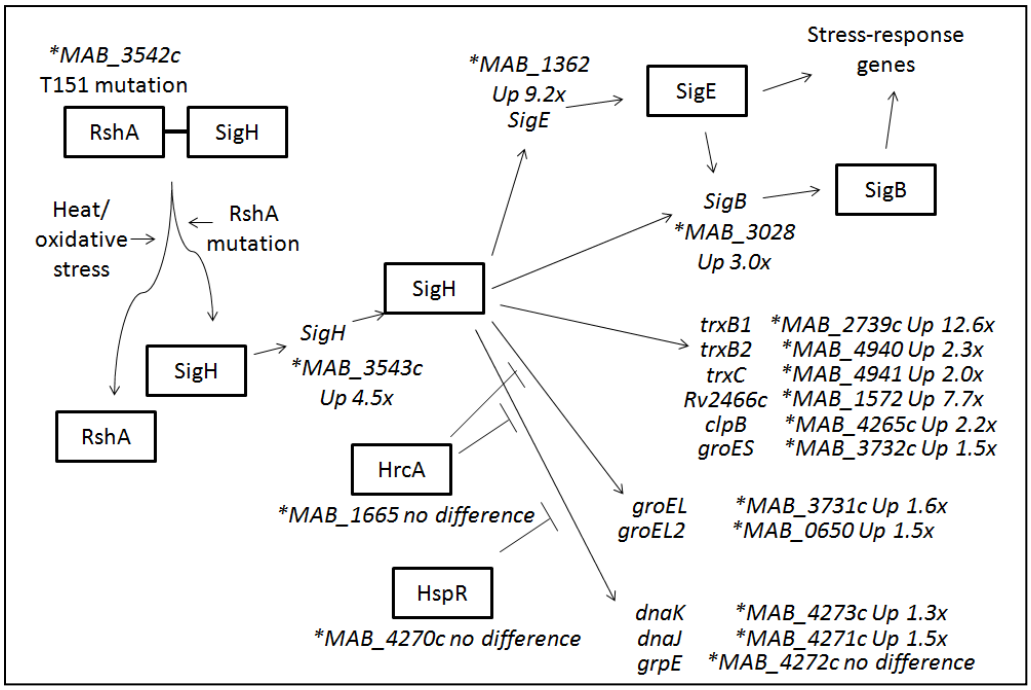

Figure 4.10: SigH-dependent stress-response pathway in *Mycobacterium tuberculosis*. Interaction between SigH and RshA can be disrupted by heat/oxidative stress or mutations in RshA, leading to the release of SigH. The released SigH in turn binds to the core RNA polymerase, leading to auto up-regulation of *sigH* and increased transcription of genes encoding other sigma factors and downstream stress-response genes. The orthologous genes of *M. absecssus* (identified by BLASTp) along with the fold difference in expression between 7C and ATCC 19977 (from RNA-Seq) were indicated by asterisks (*). The *MAB_3542c* T151 mutation was found in 7C. Refer to Table 4.8 for more details on the BLAST and RNA-Seq statistics.

Table 4.8: Genes involved in the *Mycobacterium tuberculosis* (*Mtb*) and *M. abscessus* (*Mab*) SigH-dependent stress response pathway.

***Mtb* gene**		***Mab* ortholog**							**RNA-Seq (7C vs ATCC 19977)**	
Gene	**Gene product**	**Gene**	**Gene product**	**Score**	**E-value**	**Coverage (%)**	**Identity (%)**	**Positive (%)**	**q-value**	**Fold change**
Rv3223c	SigH/RpoE	*MAB_3543c*	RNA polymerase sigma-E factor	343	5×10^{-122}	89	84	91	<0.01	4.5
Rv1221	SigE	*MAB_1362*	Probable alternative RNA polymerase sigma factor	333	4×10^{-117}	90	73	80	<0.01	9.2
Rv2710	SigB	*MAB_3028*	RNA polymerase sigma factor	567	0	100	86	93	<0.01	3.0
Rv1471	TrxB1	*MAB_2739c*	Probable thioredoxin TrxB	166	3×10^{-55}	90	68	81	<0.01	12.6
Rv3913	TrxB2	*MAB_4940*	Thioredoxin reductase (TrxB)	459	2×10^{-164}	91	76	87	<0.01	2.3
Rv3914	TrxC	*MAB_4941*	Thioredoxin (Trx)	165	2×10^{-55}	91	75	83	<0.01	2.0
Rv2466c	Rv2466c	*MAB_1572*	Hypothetical protein	339	4×10^{-121}	98	79	86	<0.01	7.7
Rv0384c	ClpB	*MAB_4265c*	Chaperone ClpB	1458	0	100	86	93	<0.01	2.2
Rv3418	GroES	*MAB_3732c*	10 kDa chaperonin (GroES)	189	2×10^{-65}	97	98	98	<0.01	1.5
Rv3417c	GroEL	*MAB_3731c*	60 kDa chaperonin 1 (GroEL protein 1)	786	0	98	78	88	<0.01	1.6
Rv0440	GroEL2	*MAB_0650*	60 kDa chaperonin 2 (Protein Cpn60 2) (GroEL)	979	0	100	94	95	0.01	1.5

The *Mab* orthologs were identified using BLASTp (BLAST statistics were included). The results of differential gene expression analysis (7C versus ATCC 19977) were included. *7C harbored the T151C mutation in *MAB_3542c*; N/A: Not applicable.

Table 4.8 (continued): Genes involved in the *Mycobacterium tuberculosis* (*Mtb*) and *M. abscessus* (*Mab*) SigH-dependent stress response pathway.

***Mtb* gene**		***Mab* ortholog**							**RNA-Seq (7C vs ATCC 19977)**	
Gene	**Gene product**	**Gene**	**Gene product**	**Score**	**E-value**	**Coverage (%)**	**Identity (%)**	**Positive (%)**	**q-value**	**Fold change**
Rv0350	DnaK	*MAB_4273c*	Chaperone protein DnaK (Hsp 70)	1097	0	100	89	94	0.03	1.3
Rv0352	DnaJ	*MAB_4271c*	Chaperone protein DnaJ	536	0	100	78	86	<0.01	1.5
Rv0351	GrpE	*MAB_4272c*	Protein GrpE (HSP-70 cofactor)	206	8×10^{-68}	74	58	67	0.18	N/A
Rv2374c	HrcA	*MAB_1665*	Heat-inducible transcription repressor HrcA	549	0	100	81	90	0.09	N/A
Rv0353	HspR	*MAB_4270c*	Probable heat shock protein transcriptional regulator HspR	184	2×10^{-62}	97	76	87	0.20	N/A
Rv3221A	RshA	*MAB_3542c**	Conserved hypothetical protein (possible anti-sigma factor)	121	2×10^{-38}	76	70	88	0.23	N/A

The *Mab* orthologs were identified using BLASTp (BLAST statistics were included). The results of differential gene expression analysis (7C versus ATCC 19977) were included. *7C harbored the T151C mutation in *MAB_3542c*; N/A: Not applicable.

4.9.2 Down-regulated genes

A myriad of ribosomal protein-encoding genes were down-regulated in 7C (Table 4.9). Among the under-expressed, ribosomal protein-encoding genes, *rpsJ* is of particular interest because mutations found in this gene have been linked to decreased tigecycline susceptibility in other bacterial genera (Beabout et al. 2015; Lupien et al. 2015).

On the other hand, pyruvate was the most enriched function among the down-regulated genes. The mapped genes included those encoding different components of the pyruvate dehydrogenase complex. The expression of genes encoding various lipoproteins was also found to be lower in 7C. In addition, the genes encoding putative resuscitation-promoting factors, *MAB_0869c* and *MAB_1130* (mapped to the function term: signal), were found to be under-expressed in the mutant.

Table 4.9: Over-represented functions (p-value <0.05) in down-regulated genes of *Mycobacterium abscessus* 7C (q-value <0.05, fold change ≤-1.5).

Gene	Gene product	q-value	Fold change
Pyruvate (fold enrichment = 6.0)			
MAB_0896c	Putative pyruvate dehydrogenase E1 component, beta subunit	<0.01	-2.7
MAB_0895c	Putative dihydrolipoamide s-acetyltransferase component of pyruvate dehydrogenase complex E2	<0.01	-2.9
MAB_0897c	Probable pyruvate dehydrogenase E1 component, alpha subunit	<0.01	-3.0
MAB_0893	4-hydroxyphenylpyruvate dioxygenase	<0.01	-2.4
Ribosomal protein (fold enrichment = 5.3)			
rpsT	30S ribosomal protein S20	<0.01	-1.9
rpmA	50S ribosomal protein L27	<0.01	-1.6
rpsO	30S ribosomal protein S15	<0.01	-1.8
rpsL	30S ribosomal protein S12	<0.01	-1.8
rpsJ	30S ribosomal protein S10	<0.01	-1.5
rplS	50S ribosomal protein L19	<0.01	-1.8
rplM	50S ribosomal protein L13	0.01	-1.6
rpmB	50S ribosomal protein L28	<0.01	-2.0
rplY	50S ribosomal protein L25/general stress protein Ctc	<0.01	-1.6
rplN	50S ribosomal protein L14	<0.01	-1.5
rpsI	30S ribosomal protein S9	<0.01	-1.5
rpmF	50S ribosomal protein L32	<0.01	-1.9
Lipoprotein (fold enrichment = 3.8)			
MAB_1145c	Putative lipoprotein LpqT precursor	<0.01	-1.5
MAB_2379	Hypothetical lipoprotein LpqH precursor	<0.01	-2.2
MAB_3107c	Possible lipoprotein LppU	<0.01	-2.1
MAB_0885c	Hypothetical lipoprotein lpqH precursor	<0.01	-1.5
MAB_3225	Putative lipoprotein LppW precursor	<0.01	-1.6
MAB_1402c	Putative lipoprotein LprE precursor	<0.01	-1.6
RNA-binding (fold enrichment = 3.4)			
rpsT	30S ribosomal protein S20	<0.01	-1.9
rpsO	30S ribosomal protein S15	<0.01	-1.8
rpsL	30S ribosomal protein S12	<0.01	-1.8
MAB_1131	16S rRNA (adenine(1518)-N(6)/adenine(1519)-N(6))-dimethyltransferase	<0.01	-1.8
rnpA	Ribonuclease P protein component	<0.01	-1.5
rplY	50S ribosomal protein L25/general stress protein Ctc	<0.01	-1.6
rplN	50S ribosomal protein L14	<0.01	-1.5

Functions were ranked according to the fold enrichment. ∞ indicates that the gene was expressed in *M. abscessus* ATCC 19977 but not in 7C.

Table 4.9 (continued): Over-represented functions (p-value <0.05) in down-regulated genes of *Mycobacterium abscessus* 7C (q-value <0.05, fold change ≤-1.5).

Gene	Gene product	q-value	Fold change
Signal (fold enrichment = 2.2)			
MAB_1596	Hypothetical protein	0.02	-1.5
MAB_3107c	Possible lipoprotein LppU	<0.01	-2.1
MAB_1261	Hypothetical protein	<0.01	-4.7
MAB_4791c	Hypothetical protein	<0.01	∞
MAB_2972	Hypothetical protein	<0.01	-2.0
MAB_3249	Hypothetical protein	<0.01	-1.5
MAB_1263	Hypothetical protein	<0.01	-6.5
MAB_2422c	Hypothetical protein	<0.01	-1.7
MAB_0869c	Probable resuscitation-promoting factor RpfA	<0.01	-2.1
MAB_4461	Hypothetical protein	<0.01	-1.5
MAB_2903	Hypothetical protein	0.02	-19.3
MAB_0848c	Hypothetical protein	<0.01	-1.9
MAB_1608c	Hypothetical protein	<0.01	-1.8
MAB_2329c	Hypothetical protein	<0.01	-2.0
MAB_1652	Probable sulfate ABC transporter, sulfate-binding protein SubI	0.03	-1.6
MAB_4062c	Hypothetical protein	<0.01	-1.6
MAB_1026c	Hypothetical protein	<0.01	-1.8
MAB_4133c	Hypothetical protein	<0.01	-1.5
MAB_4903	Hypothetical protein	<0.01	-1.8
MAB_1130	Hypothetical protein	<0.01	-1.7

Functions were ranked according to the fold enrichment. ∞ indicates that the gene was expressed in *M. abscessus* ATCC 19977 but not in 7C.

4.10 Biological verification with RT-qPCR

Based on the RNA-Seq profiling, the up-regulation of *MAB_3543c* (Table 4.7) and down-regulation of *rpsJ* were detected in 7C (Table 4.9). The mRNA levels of these genes were then quantified in 7C-*MAB_3542c*^{wt} and 7C-pMV261 using reverse-transcriptase real-time PCR (RT-qPCR). *MAB_3543c*

and *rpsJ* were selected for verification based on their biological significance (see section 4.9.1 and 4.9.2). Complementation of 7C with the plasmid pMV261 carrying the wt *MAB_3542c* significantly (a) down-regulated the expression of *MAB_3543c* (p-value <0.01, fold change = -5.8) (Figure 4.11A) and (b) up-regulated the expression of *rpsJ* (p-value = 0.04, fold change = 1.1) (Figure 4.11B). This signified that the expression changes of these two genes observed in 7C were likely a consequence of *MAB_3542c* mutation.

4.11 Heat-inducible tigecycline resistance

The RNA-Seq analysis demonstrated that the *MAB_3542c* mutation, a determinant of tigecycline resistance (see section 4.5), may potentially activate the heat/oxidative stress response mechanism (Figure 4.10). Based on these findings, a new hypothesis was formulated: tigecycline resistance may be heat-inducible in *Mab*. In agreement with this, exposure of wt ATCC 19977 cells at 42 ℃ for 1 hour predisposed the organism to better withstand the subsequent tigecycline challenge (8 mg/L) (Figure 4.12). However, with the agar disk diffusion test which requires at least three days to develop an inhibition zone for *Mab*, no significant change in tigecycline susceptibility pattern was detected between the wt cells pre-treated at 42 ℃ and at 37 ℃ for 1, 4, and 8 hours (zone diameter of cells pre-exposed to 42 ℃ for 1 hour: 35 mm; 2 hours: 36 mm; 8 hours: 35 mm and zone diameter of cells pre-exposed to 37 ℃ for 1 hour: 34 mm; 2 hours: 36 mm; 8 hours: 36 mm).

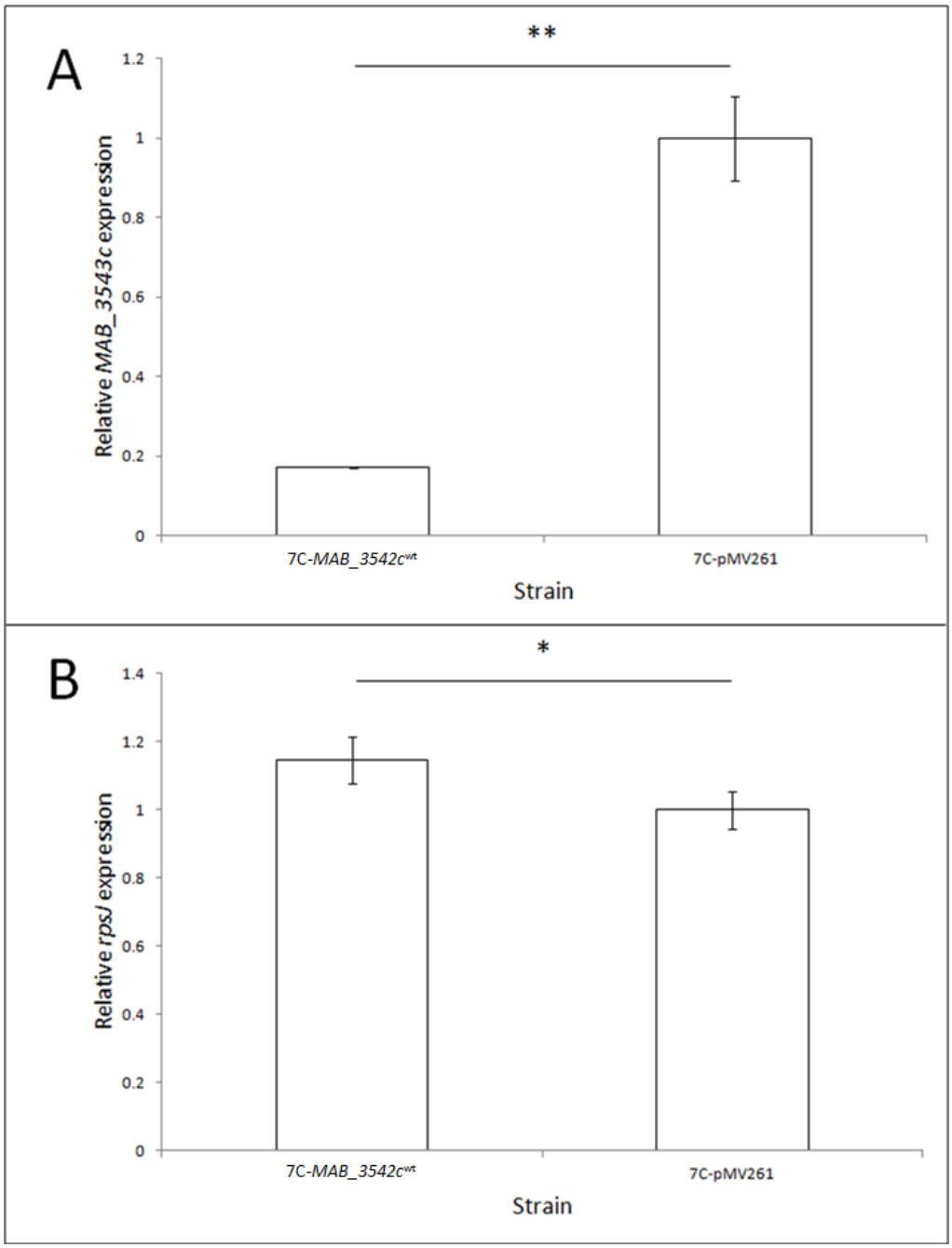

Figure 4.11: Expression levels of (A) *MAB_3543c* and (B) *rpsJ* in 7C-*MAB_3542c*^{wt} and 7C-pMV261, measured by RT-qPCR. Data were expressed as mean ± standard deviation of biological triplicates. *p-value <0.05; **p-value <0.01.

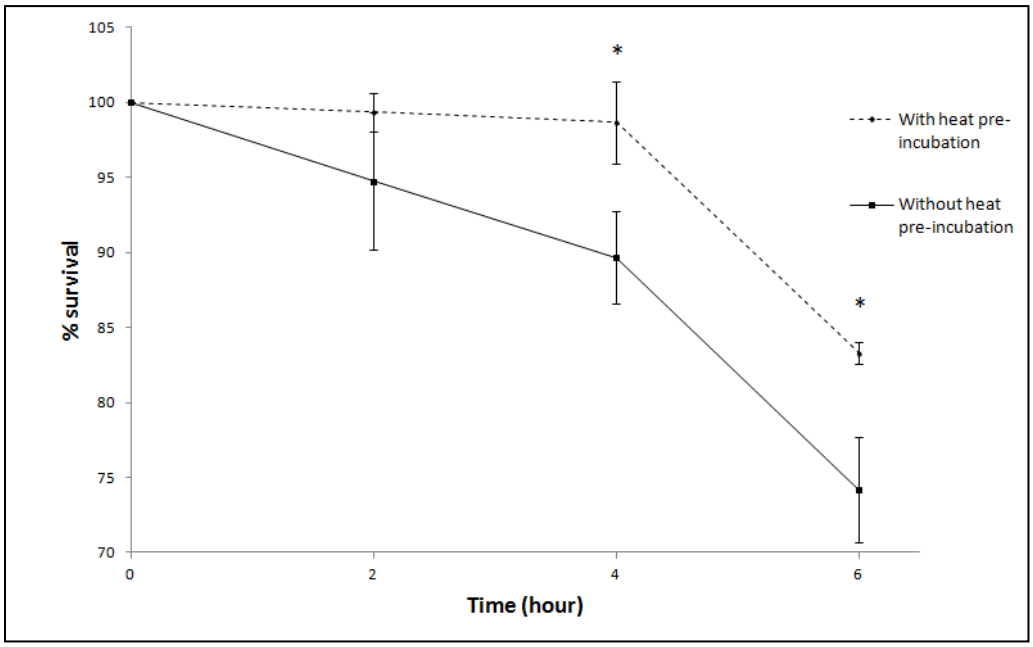

Figure 4.12: Survival of *Mycobacterium abscessus* ATCC 19977, with and without pre-treatment at 42 ℃, in broth containing 8 mg/L tigecycline. Data were expressed as mean ± standard deviation of biological triplicates. *p-value <0.05.

CHAPTER 5

DISCUSSION

5.1 *MAB_3542c* T151C mutation was a molecular determinant of tigecycline resistance in *Mycobacterium abscessus*

In this study, 7C, a tigecycline-resistant mutant of *Mab* ATCC 19977, was characterized (see section 4.1 to 4.4). Compared to the wt, 7C demonstrated resistance to tigecycline (Figure 4.1 and Table 4.1) as well as cross-resistance to imipenem (Figure 4.1 and Table 4.1), and had a slightly retarded growth rate (Figure 4.3). WGS (see section 4.4) and subsequent complementation experiments (see section 4.5) showed that these phenotypes were likely to be caused by a point mutation in *MAB_3542c*. Based on the BLASTp analysis, this gene may encode an RshA-like protein (Figure 4.7).

The level of antibiotic resistance conferred by a mutation can be affected by the copy number of the gene harboring the said mutation. For example, *S. pneumoniae* has four copies of *16S rRNA* gene in its genome (Kilian et al. 2008). Lupien et al. (2015) reported that the level of tigecycline resistance is positively correlated to the number of gene copies which carried the mutation in this bacterium. This implies that, in order for *S. pneumoniae* to achieve the highest level of resistance, mutations must occur in all four copies of the *16S rRNA* gene in its genome. In contrast, *Mab* was found to have one

copy of *MAB_3542c* gene (see section 4.4), thus making the bacterium susceptible to single-step mutations. This may explain why the mutation (T151C) in this gene (found in 7C) could cause a substantial increase in the level of tigecycline resistance (8 fold, from 0.25 to 2 mg/L, Table 4.1), crossing the breakpoint for resistance (1 mg/L) in just one step.

5.2 The MAB_3542c mutation might disrupt the sigma factor-anti sigma factor interaction

In *Mtb*, the sigma factor SigH is a central regulator of heat- and oxidative-stress response while RshA is an anti-sigma factor which inhibits SigH-dependent transcription (Song et al. 2003). At an elevated temperature or in oxidizing conditions, interaction between SigH and RshA is disrupted, leading to the release of SigH. The released SigH in turn binds to the core RNA polymerase, leading to auto up-regulation of *sigH* and increased transcription of genes encoding other sigma factors and downstream stress-response genes (Song et al. 2003). Moreover, it was found that the interaction of RshA and SigH can also be disrupted by a mutation in either one of the cysteine residues in the HXXXCXXC motif of RshA (Song et al. 2003). Interestingly, the *MAB_3542c* mutation found in 7C altered the first cysteine residue in this motif to arginine (Figure 4.7B). Hence, this may lead to the activation of MAB_3543c (the ortholog of SigH in *Mab*) and over-transcription of stress-response genes in 7C, with or without external stress signals (e.g. heat). It is well-documented that bacterial stress responses are determinants of

antimicrobial resistance (Poole 2012). For example, Tran et al. (2011) reported that a heat shock protein (ClpL) in *S. pneumoniae* is able to modulate cell wall biosynthetic enzymes, which leads to decreased penicillin susceptibility. Whole-transcriptome profiling of 7C was warranted to identify the dysregulated genes and pathways caused by the mutation in *MAB_3542c* (see section 4.9).

Anti-sigma factors have previously been implicated in antimicrobial resistance. For example, a recent study demonstrated that the deletion of a putative anti-sigma factor gene (*MSMEG_6129*) could lead to chloramphenicol, isoniazid, and tetracycline resistance in *M. smegmatis* and subsequent complementation of this deletion mutant with exogenous *MSMEG_6129* restored the sensitive phenotype to the bacterium (Bowman & Ghosh 2014). In good agreement with their findings, it had been demonstrated in this study that a non-mutated and functional anti-sigma factor (MAB_3542c) is needed to maintain the tigecycline susceptibility in *Mab*. Interestingly, orthologs of the MSMEG_6129 protein were not found in the *Mab* proteome, as the BLASTp analysis did not identify any *Mab* proteins which satisfied the cutoff threshold for the ortholog search (see section 3.7.1). This suggests that the MAB_3542c mutation may be a novel determinant of antibiotic resistance in *Mycobacterium.*

5.3 Transcriptome profiling revealed dysregulation of stress-response genes in 7C

7C was subjected to transcriptomic dissection using RNA-Seq. Compared to ATCC 19977, various functions were found to be affected in 7C (Table 4.7 and 4.9). Sigma factors are important bacterial transcription activators that attach to the RNA polymerase and provide the specificity for certain promoters (Missiakas & Raina 1998). This provides a way for bacterial cells to regulate the expression of specific genes in response to various environmental conditions, including stresses. The interaction of RshA (*Mab* ortholog: MAB_3542c) and SigH (*Mab* ortholog: MAB_3543c) can be disrupted by elevated temperature, oxidative stress, or a mutation in either one of the cysteine residues in the HXXXCXXC motif of RshA (Song et al. 2003). Consequently, this disruption releases SigH to bind to the core RNA polymerase, leading to auto up-regulation of *sigH* and increased transcription of genes encoding other sigma factors, SigB and SigE (Fernandes et al. 1999; Raman et al. 2001). In line with this observation, *MAB_3543c*, *MAB_1362* (*Mtb* ortholog: *SigE*), and *MAB_3028* (*Mtb* ortholog: *SigB*) were among the over-expressed genes of 7C that mapped to the sigma factor function (Table 4.7). In addition, the chaperone-encoding genes were also found to be up-regulated in 7C (Table 4.7). This may explain the phenotype showing improved survival of 7C against heat shock (Figure 4.9). *MAB_3543c* was shown to be down-regulated upon complementation of 7C with the wt *MAB_3542c* (Figure 4.11A), suggesting a possible connection between *MAB_3542c* mutation and dysregulation of *MAB_3543c* in 7C.

In *Mtb*, RshA dissociation and the subsequent SigH activation can be induced by redox imbalance, in addition to heat-stress signals (Song et al. 2003). In 7C, genes encoding various oxidoreductases, such as thioredoxin, catalase, luciferase/luciferase-like protein, etc, were over-expressed (Table 4.7). In 2003, Szpilewska et al. showed that bacterial luciferase plays a physiological role in protecting the cells from oxidative stress. Meanwhile, *MAB_2530c* encodes the catalase CatB, which has recently been shown to have protective effect against reactive oxygen species (ROS) for *Xanthomonas oryzae*, a rice pathogen (Pan et al. 2017). Thioredoxins are oxidoreductases which perform the thiol-disulfide exchange reaction and are crucial for the maintenance of redox homeostasis in bacterial cells (Reniere 2018). Interestingly, MAB_2739c and MAB_4940 are potential orthologs of *Mtb* TrxB1 and TrxB2, respectively (Table 4.8). The transcription of these thioredoxin-encoding genes was shown to be induced by heat and oxidative stress through SigH activation in *Mtb* (Raman et al. 2001; Song et al. 2003).

In the present study, orthologous genes of *Mtb* genes that are directly under the regulation of SigH (Raman et al. 2001; Mangan et al. 2002; Song et al. 2003; Manganelli et al. 2002) were identified in *Mab* using BLASTp (Table 4.8). Interestingly, all genes mapped to the SigH pathway were significantly up-regulated in 7C, with a lone exception of *MAB_4272c* (Figure 4.10 and Table 4.8). In addition, no difference in expression was observed for genes encoding orthologs of the transcriptional repressors HrcA (*MAB_1665*) and HspR (*MAB_4270c*) (Figure 4.10), supporting the up-regulation of several heat-shock response genes, such as *groEL*, *groEL2*, *dnaK*, and *dnaJ*, in the

mutant 7C. However, *grpE* (*Mab* orthologous gene: *MAB_4272c*), which is under the regulation of HrcA and HspR in *Mtb* (Mangan et al. 2002), was not differentially expressed between 7C and ATCC 19977 (Figure 4.10). This signified that an alternative mechanism may be involved in the regulation of *MAB_4272c* in *Mab*. Taken together, these findings are in good agreement with the hypothesis that *MAB_3542c* mutation induces heat/oxidative stress responses through MAB_3543c activation, even in the absence of stress stimuli.

A recent study showed that elevated temperature down-regulates genes involved in the pyruvate consumption to promote the accumulation of pyruvate, a molecule that scavenges heat-induced ROS (Zhang et al. 2017). Interestingly, genes encoding different components of the pyruvate dehydrogenase complex were under-expressed in the mutant (Table 4.9), possibly as a protective mechanism against cellular damages stimulated by stress.

Bacteria encounter many stresses in their natural environments. If a mutant like 7C exists in the environment, the enhanced stress response caused by the MAB_3542c mutation may confer selective advantages for the survival of the bacterium under harsh environmental conditions and hence, the antibiotic-resistance determinant may be retained through evolution. This may eventually lead to the occurrence of (a) horizontal transfer of the antibiotic-resistance to other bacteria and (b) infections which are resistant to antibiotic treatment.

5.4 Down-regulation of ribosomal protein-encoding genes in 7C might contribute to phenotypic resistance to tigecycline

The deletion of the WhiB7-encoding gene (*MAB_3508c*) has previously been shown to be associated with reduced tigecycline susceptibility in *Mab* (Pryjma et al. 2017). In the present study, this gene was not differentially expressed in 7C comparing to ATCC 19977 (q-value = 0.82). This suggests that an alternative, WhiB7-independent pathway may be required to cause tigecycline resistance in 7C.

An interesting observation from the RNA-Seq analysis is that many ribosomal protein-encoding genes were down-regulated in 7C (Table 4.9). Research has shown that exposure to elevated temperature decreases the gene expression of bacterial ribosomal proteins, possibly as an adaptation to counter the stress-induced damage (Zengel & Lindahl 1985). It is possible that the MAB_3542c mutation might trigger the stress response mechanisms, causing the down-regulation of *rpsJ* and other genes encoding 30S ribosomal proteins. The down-regulation of these genes might cause changes in the conformation and the assembly of ribosomes, leading to tigecycline resistance in 7C. This is supported by the down-regulation of *MAB_1131*, which encodes a 16S rRNA dimethyltransferase (mapped to the function of RNA binding) (Table 4.9); studies have shown that this methyltransferase plays a crucial role in establishing a proper conformation of the 30S subunit during the ribosome biogenesis (Connolly et al. 2008; Demirci et al. 2010). The *rpsJ* dysregulation was reverted upon complementation of 7C with the wt *MAB_3542c* (Figure

4.11B), indicating that the dysregulation of *rpsJ* observed in 7C is likely an outcome of the *MAB_3542c* mutation. It may be interesting to characterize the tigecycline binding capacity of the ribosomes isolated from 7C in future studies. Furthermore, the MAB_3542c mutation might impart a fitness cost to the bacterium by affecting the protein-synthesis apparatus, causing the mutant to replicate at a slower rate than its parental strain in the absence of tigecycline (Figure 4.3). It would be interesting to see how the mutation can affect the fitness of 7C to establish an infection in animal models.

5.5 Down-regulation of virulence factor-encoding genes in 7C

Several genes which confer bacterial virulence, such as those encoding lipoproteins and resuscitation-promoting factors, were also down-regulated in 7C (Table 4.9). Bacterial lipoproteins are membrane proteins with many different functions. Studies have shown that lipoproteins can positively influence the *in vivo* virulence and survival of mycobacteria (Sánchez et al. 2012; Li et al. 2018). Kana et al. (2008) demonstrated that resuscitation-promoting factors are required for virulence and resuscitation of *Mtb* from dormancy. While it is unclear how the MAB_3542c mutation would lead to the attenuation of lipoproteins and resuscitation-promoting factors, it would be interesting to study how these changes could impact the fitness of 7C to establish an infection in animal models.

5.6 Transient tigecycline resistance might be heat-inducible in *Mycobacterium abscessus*

Consolidating the findings from both phenotypic and genotypic characterizations of 7C, it was found that tigecycline resistance might be heat-inducible in *Mab*. In line with this hypothesis, a time-kill kinetic assay demonstrated that the exposure of ATCC 19977 cells to heat predisposed the organism to better resist tigecycline killing (Figure 4.12). This observation suggested that tigecycline might be less efficacious against *Mab* infections in patients with fever. However, the agar disk diffusion test failed to reproduce this phenomenon, as no significant change in tigecycline susceptibility pattern was detected between the wt cells pre-treated at 42 ℃ and at 37 ℃ (see section 4.11). A possible explanation for this failure to demonstrate heat-induced resistance with the disk diffusion test could be that the effect of heat shock on tigecycline susceptibility is transient, and thus, is not observable in the disk diffusion test that requires at least three days of incubation to develop an inhibition zone for *Mab*. Other than heat stress, RshA is also able to detect redox imbalance (Song et al. 2003) and hence, it is likely that tigecycline resistance can also be induced by oxidizing agents. This aspect, however, was not pursued in this study.

5.7 Over-expression of *MAB_3542c* failed to increase the level of tigecycline resistance in ATCC 19977

In the present study, a gene-dosage study was attempted to over-express *MAB_3542c*^{wt} in the wt background (see section 4.5). However, this did not lead to an increase in tigecycline resistance when compared to the empty-plasmid control (Table 4.4). Under normal and non-stressful conditions, the anti-sigma factor RshA binds and inhibits the sigma factor SigH in *Mtb* (Song et al. 2003). In good agreement with this finding, RNA-Seq profiling in this study revealed that the gene expression of *MAB_3542c* (anti-sigma factor) was 1.9-fold higher than that of *MAB_3543c* (sigma factor) in the tigecycline-sensitive ATCC 19977 (p-value <0.01). Since the transcripts of the anti-sigma factor were present in excess, this may explain why the over-expression of *MAB_3542c*^{wt} was unable to alter the level of resistance in the wt bacterium. On the contrary, in the tigecycline-resistant 7C, the gene expression of *MAB_3542c* (with T151C mutation) was 1.5-fold lower than that of *MAB_3543c* (p-value <0.01). Hence, these findings suggest that, instead of over-expression of wt *MAB_3542c,* ATCC 19977 is more likely to become resistant through gene knockdown/knockout of wt *MAB_3542c* or over-expression of wt *MAB_3543c*.

5.8 The mutant 7C was cross-resistant to imipenem, a more commonly used antibiotic

Imipenem is one of recommended drugs for the treatment of infections caused by members of the *Mab* complex (Lee et al. 2015). The development of cross-resistance to imipenem in 7C (Figure 4.1 and Table 4.1) highlights the possibility of tigecycline resistance (conferred by the *MAB_3542c* mutation) to be co-selected by the more commonly used imipenem in the clinical setting. However, it is unclear how the MAB_3542c mutation can lead to the imipenem cross-resistance in *Mab*. Several putative beta-lactamase-encoding genes were found to be up-regulated in 7C (see section 4.9.1) but it is unknown whether these proteins are able to catalyze the hydrolysis of imipenem. Therefore, a follow-on study is proposed to over-express these target genes in the wt ATCC 19977 to further explore their role in resistance to imipenem or other carbapenems.

Although the *MAB_3542c* mutation is likely to affect the resistance level of tetracyclines (e.g. tetracycline and doxycycline) as well, it was not possible to show this change with the disk diffusion test because there was no tetracycline inhibition zone for both 7C and ATCC 19977 (Table 4.1). An MIC determination was required to demonstrate any increase in tetracycline resistance but, unfortunately, neither the Etest nor broth microdilution for tetracycline was available in this study.

5.9 The MAB_3542c mutation might not be clinically relevant

In the present study, 48 patient isolates of *Mab* complex were screened for (a) tigecycline susceptibility pattern using Stokes disk diffusion and (b) *MAB_3542c* mutation using PCR-Sanger sequencing (see section 4.7). Surprisingly, no mutation was detected in the amino acid sequences of these clinical isolates, even in those expressing a higher level of resistance than 7C, as evidenced by their growth inhibition diameters in the disk diffusion test (Table 4.6). This suggests that the MAB_3542c mutation might not be selected in the clinical setting, which could be due to the lack of exposure to tigeycline (i.e. the hosts of these clinical isolates might not have been treated with tigecycline before), as tigecycline is a relatively new antibiotic and is not commonly used to treat infection caused by members of *Mab* complex (Griffith et al. 2007). This might explain why the tigecycline-selected resistance determinant, such as the *MAB_3542c* mutation, was not observed in the patient isolates. However, with inadequate patient information, it was not possible to know whether tigecycline treatment had been given to the hosts of these clinical isolates. Hence, it is not possible to state that the occurrence of reduced susceptibility or resistance to tigecycline in some of the isolates was due to the selection of resistance under antibiotic (tigecycline) pressure. Another possible explanation to this phenomenon (i.e. the MAB_3542c mutation was not found in any of the clinical strains tested) was the fitness cost conferred by the mutation (Figure 4.3), which may affect the fitness of the bacterium to establish an infection. Hence, an interesting follow-on study would be to investigate the possibility of 7C developing compensatory mutation(s) to

negate the reduced fitness caused by the resistance mutation, to enhance its ability to survive in the *in vivo* environment.

The tigecycline-resistance phenotype observed in several patient isolates (Table 4.6) could be due to mechanisms other than the MAB_3542c mutation, which might be a consequence of co-selection of (a) resistance determinants by other more commonly used antibiotics or (b) those genetic determinants associated with enhanced fitness or virulence (Andersson 2006). Unfortunately, it was not possible to screen for these resistance determinants as genome sequences of the tigecycline-resistant clinical isolates were not available in this study.

5.10 The *MAB_3542c* gene might exhibit phylogenetic signal for *Mycobacterium abscessus* subspecies typing

The subspecies typing of *Mab* complex is not straightforward (Macheras et al. 2009; Zelazny et al. 2009) and usually requires the sequencing of multiple genes (Macheras et al. 2014), which is cumbersome and expensive. A single-gene approach that is technically less laborious and more economical would shorten the turnaround time in diagnosis. Interestingly, the *MAB_3542c* gene appears to be useful for subspecies classification of the *Mab* complex (Figure 4.8). This observation has to be confirmed with further investigation using a larger number of clinical isolates. The subdivision of *Mab* complex into two or three closely related subspecies remains a subject of constant debate

within the scientific community (Leao et al. 2011; Tortoli et al. 2016). In the present study, the *MAB_3542c*-based phylogenetic grouping supports the notion that the *Mab* complex is differentiated into three subspecies (see section 4.7).

5.11 *Mycobacterium massiliense* was more resistant to tigecycline than *M. abscessus* subspecies *abscessus*

Contrary to the popular belief that *M. massiliense* is generally more susceptible to antibiotics than the other two subspecies of *Mab* complex (Koh et al. 2011), the *M. massiliense* strains tested in the present study were significantly more resistant to tigecycline than the *Mab* strains (see section 4.7). In good agreement with this, Ananta et al. (2018) reported a significant association of high tigecycline MICs with the *M. massiliense* subspecies, indicating the potential risk of tigecycline treatment failure for this subspecies. This reiterates the importance of subspecies classification in the clinical management of infections caused by members of the *Mab* complex.

5.12 Limitations

For the interpretation of results, several aspects of this study were observational in nature. Although direct results from observational studies are known to be problematic for causal inference (Glass et al. 2013), they are

useful for generating hypotheses to be tested. Experimental validation, such as complementation, then provides the evidence for interpretation in the subject matter context.

Cost considerations limited characterization experiments to one tigecycline-resistant mutant strain of *Mab*. Since there is more than one way for bacteria to become resistant to an antibiotic, future endeavors should be made to characterize more resistant mutants of *Mab* to have a better understanding of tigecycline resistance mechanisms in this important pathogen.

It is also important to note that the findings of this study, which were based on the characterization of the laboratory-derived mutant 7C, did not accurately reflect what was observed in the clinical strains of *Mab* complex, as the MAB_3542c mutation was not found in any of these isolates (see section 4.7 and 5.9). A clinically relevant source of resistance-associated mutations would be *Mab* isolates from patients treated with tigecycline, particularly those who show treatment failure with tigecycline and thus, are very likely to be infected with tigecycline-resistant strains.

CHAPTER 6

CONCLUSION

Taken together, WGS of 7C and subsequent verifications revealed that the T151C mutation in *MAB_3542c*, which encodes a putative RshA-like anti-sigma factor, may represent a novel determinant of tigecycline resistance in *Mab*. Subsequent RNA-Seq analysis revealed that this mutation may trick the *Mab* cells to be trapped in the "defensive" mode, as demonstrated by the improved response against heat stress and over-expression of genes encoding the SigH ortholog (MAB_3543c) and various chaperones and oxidoreductases in 7C. These findings suggest that dysregulated stress response may be a possible mechanism of tigecycline resistance in *Mab*.

The development of new antimicrobial agents for multidrug-resistant pathogens, such as *Mab*, remains a crucial medical need. The findings of this study contribute to knowledge of potential tigecycline resistance mechanisms in *Mab* that may lead to not only better therapeutics for *Mab* infection but also appropriate measures to prevent, delay, or circumvent the further spread of tigecycline resistance that will shorten the useful lifespan of this antibiotic.

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APPENDIX A

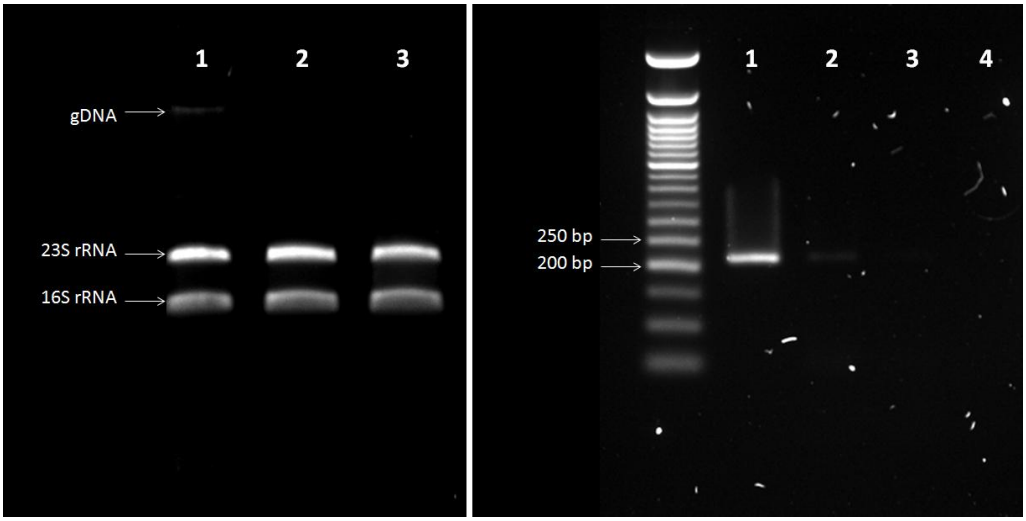
Preparation of culture media

Medium	Recipe (per L)	Remarks
Cation-adjusted Mueller-Hinton agar	N/A	Pre-poured culture media
Cation-adjusted Mueller-Hinton agar supplemented with 5 % sheep blood	N/A	Pre-poured culture media
Cation-adjusted Mueller-Hinton broth	22 g of powder	N/A
Luria-Bertani agar	40 g of powder	N/A
Luria-Bertani broth	25 g of powder	N/A
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]	N/A
Middlebrook 7H10 agar	19 g of powder 5 mL of glycerol 100 mL of Middlebrook OADC enrichment[1]	N/A
Nutrient agar	23 g of powder	N/A
Tryptic soy broth	30 g of powder	N/A

Unless otherwise stated, all agar and broth were prepared using dehydrated culture media (in powder form). Distilled water was added to a final volume of 1 L and the media were sterilized by autoclaving at 121 ℃ for 20 min. [1]Heat-labile, added after autoclaving, when the media had cooled to ~55 ℃; N/A: Not applicable.

APPENDIX B

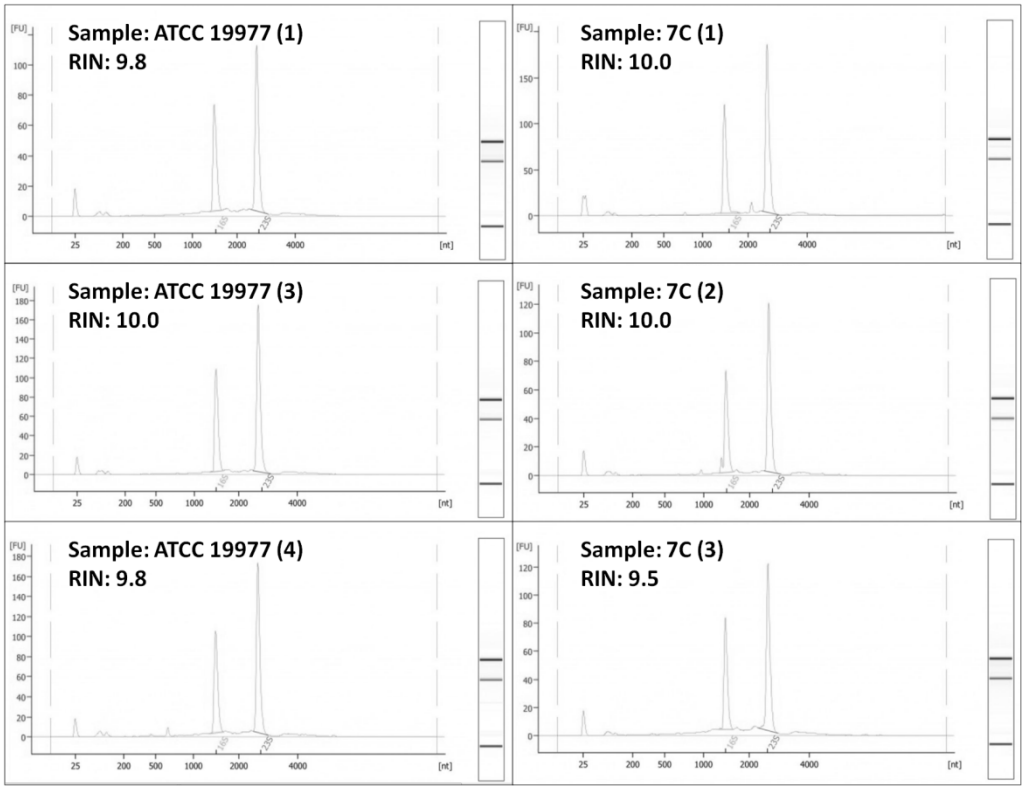
Magnitude of genomic DNA (gDNA) contamination in total RNA samples with one (1 × on-column treatment), two (1 × on-column treatment and 1 × in-solution treatment), or three (2 × on-column treatment and 1 × in-solution treatment) DNase treatments



Left panel, lane 1: RNA with one DNase treatment; lane 2: RNA with two DNase treatments; lane 3: RNA with three DNase treatments. Right panel, lane 1: PCR amplicon using RNA with one DNase treatment as template; lane 2: PCR amplicon using RNA with two DNase treatments as template; lane 3: PCR amplicon using RNA with three DNase treatments as template; lane 4: No-template control. PCR was performed using 3542-F and 3542-R primers (Table 3.3); 50 ng of RNA sample was used as template.

APPENDIX C

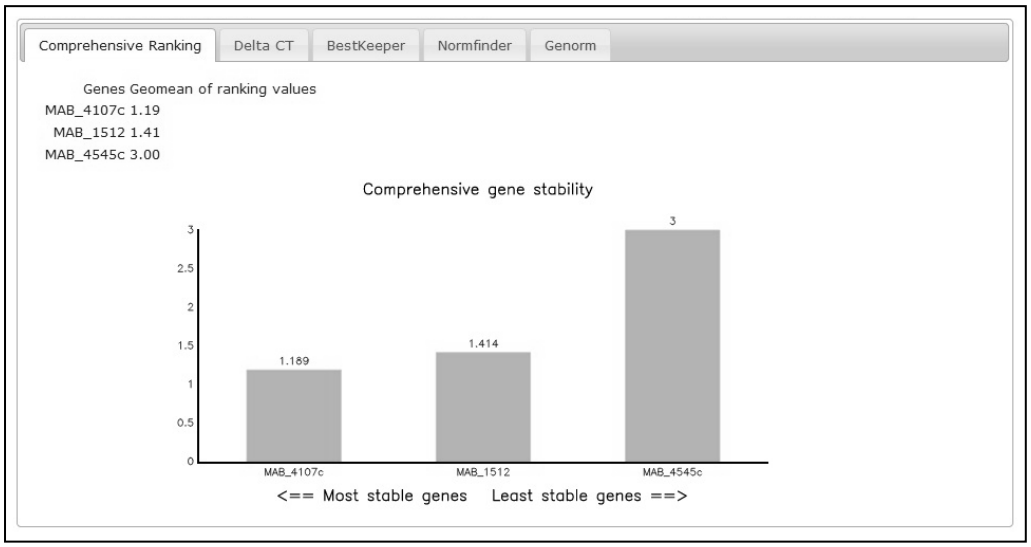
Integrity of the RNA-Seq samples, as interpreted by Agilent Bioanalyzer



A RNA integrity number (RIN), which ranges from 1 (totally degraded) to 10 (intact), was assigned to each electropherogram by the Bioanalyzer algorithm.

APPENDIX D

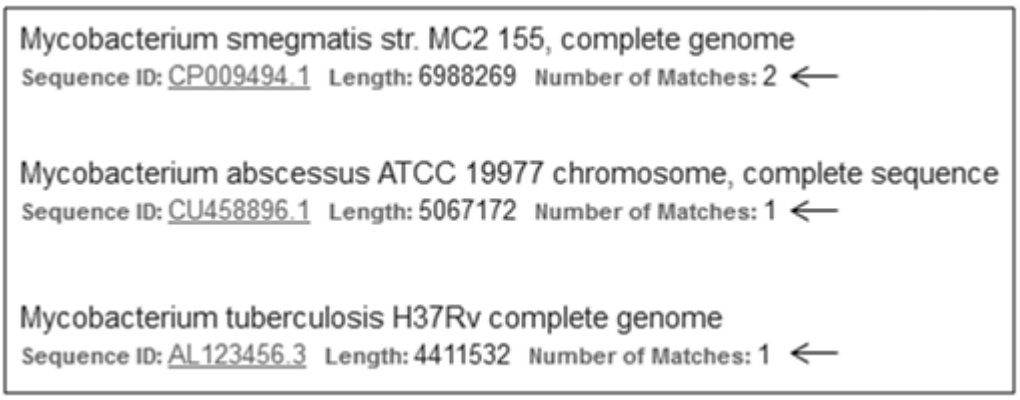
Identification of the most stably expressed gene by RefFinder



By integrating the major algorithms, including BestKeeper (Pfaffl et al. 2004), geNorm (Vandesompele et al. 2002), Normfinder (Andersen et al. 2004), and the comparative delta-Ct method (Silver et al. 2006), the candidate reference genes were compared and ranked by RefFinder based on the rankings assigned by each algorithm.

APPENDIX E

Copy numbers of *16S rRNA* gene of *Mycobacterium smegmatis*, *M. abscessus*, and *M. tuberculosis*, identified by BLASTn analysis



The copy numbers are indicated by arrows.

APPENDIX F

Antimicrobial susceptibility patterns of *Staphylococcus aureus* ATCC 29213 and ATCC 25923 (as controls) using disk diffusion

Antibiotic	Disc potency (µg)	Zone size (mm)			
		ATCC 29213	EUCAST range	ATCC 25923	BD range
Amikacin	30	22.0 ± 2.0	18-24	-	-
Chloramphenicol	30	22.7 ± 0.6	20-28	-	-
Ciprofloxacin	5	23.0 ± 1.0	21-27	-	-
Clarithromycin	15	-	-	28.3 ± 2.5	26-32
Clindamycin	2	24.7 ± 0.6	23-29	-	-
Doxycycline	30	-	-	28.3 ± 0.6	23-29
Imipenem	10	40.3 ± 1.5	-	43.0 ± 1.0	-
Linezolid	30	-	-	29.3 ± 3.1	25-32
Tetracycline	30	23.3 ± 0.6	23-31	-	-
Tigecycline	15	22.3 ± 0.6	19-25	-	-

The experiment was performed in triplicate and data were expressed as mean ± standard deviation. EUCAST: European Committee on Antimicrobial Susceptibility Testing; BD: Becton Dickinson (the manufacturer of the antibiotic-impregnated discs used in this study).

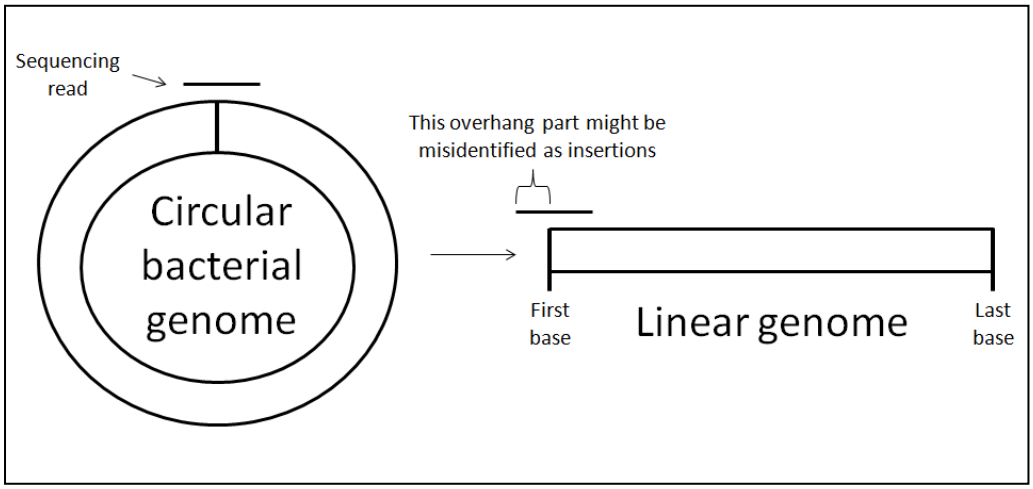
APPENDIX G

Antimicrobial susceptibility patterns of *Staphylococcus aureus* ATCC 29213 and ATCC 25923 (as controls) using Etest and broth microdilution

Antibiotic	Method	MIC (mg/L)		
		ATCC 29213	MIC range	Reference
Imipenem	Etest	0.03	0.03-0.06	Boyce et al. 1991
Tigecycline	Broth microdilution	0.25	0.03-0.25	EUCAST 2018

APPENDIX H

Multiple insertions found in *MAB_0001* might be due to reference assembly errors



Sequencing reads generated from the junction between the first and last bases of the circular bacterial genome are likely to cause assembly errors during the reference mapping.

APPENDIX I

Significantly up-regulated genes (q-value <0.05) in 7C

Gene	Gene product	q-value	Fold change
MAB_0694	Hypothetical protein	<0.01	37.5
MAB_3358c	Putative acyltransferase	<0.01	31.5
MAB_3359c	Hypothetical protein	<0.01	27.1
MAB_3016c	Hypothetical protein	<0.01	27.0
MAB_4135c	Hypothetical protein	<0.01	25.4
MAB_4143c	Putative anti-ECFsigma factor, ChrR	<0.01	23.4
MAB_1528c	Probable oxidoreductase	<0.01	21.2
MAB_4908c	Putative luciferase-like oxidoreductase	<0.01	20.8
MAB_1117c	Hypothetical protein	<0.01	19.2
MAB_3464	Hypothetical protein	<0.01	16.1
MAB_3403c	Hypothetical protein	<0.01	15.7
MAB_4234c	Putative FMNH2-utilizing oxygenase	<0.01	15.4
MAB_4748c	Conserved hypothetical protein (pyridoxamine 5'-phosphate oxidase?)	<0.01	14.6
MAB_3931c	Probable glycosyl transferase	<0.01	14.5
MAB_3377	Hypothetical protein	<0.01	13.2
MAB_3427	Hypothetical protein	<0.01	12.7
MAB_3015	Hypothetical protein	<0.01	12.7
MAB_2739c	Probable thioredoxin TrxB	<0.01	12.6
MAB_2798c	Hypothetical protein	<0.01	12.5
MAB_1339	Putative monooxygenase (luciferase-like)	<0.01	12.4
MAB_3461c	Hypothetical protein	<0.01	12.0
MAB_4735	Putative starvation-induced DNA protecting protein/Ferritin and Dps	<0.01	11.4
MAB_4295c	Probable UDP-glucose 6-dehydrogenase (UdgA)	<0.01	9.4
MAB_1362	Probable alternative RNA polymerase sigma factor	<0.01	9.2

APPENDIX I

Significantly up-regulated genes (q-value <0.05) in 7C (continued)

Gene	Gene product	q-value	Fold change
MAB_0746	Putative phosphate ABC transporter, phosphate-binding protein	<0.01	9.1
MAB_3639	Putative luciferase-like protein	<0.01	8.8
MAB_2904	Probable pyridoxamine 5'-phosphate oxidase	<0.01	8.3
MAB_0087c	Probable alkaline phosphatase	<0.01	8.3
MAB_1572	Hypothetical protein	<0.01	7.7
MAB_4746	Putative membrane protein, MmpL	<0.01	7.2
MAB_3950c	Probable low-affinity inorganic phosphate transporter	<0.01	6.9
MAB_1116	Hypothetical protein	<0.01	6.9
MAB_2905c	Hypothetical protein	<0.01	6.7
MAB_0827	Hypothetical short-chain dehydrogenase/reductase	<0.01	6.6
MAB_1299c	Putative multi-drug efflux transporter	<0.01	6.3
MAB_3949c	Hypothetical protein	<0.01	5.9
MAB_2886c	Hypothetical protein	<0.01	5.9
MAB_1486c	Putative oxidoreductase	<0.01	5.5
MAB_3465	Putative sulfate transporter/antisigma-factor	<0.01	5.4
MAB_0830	Probable NADH-dependent flavin oxidoreductase	<0.01	5.1
MAB_4233c	Putative monooxygenase (luciferase-like)	<0.01	5.0
MAB_2800	Hypothetical protein	<0.01	4.9
MAB_4048c	Sensor-like histidine kinase senX3	<0.01	4.6
MAB_2738c	Hypothetical protein	<0.01	4.5
MAB_4232c	Putative oxygenase	<0.01	4.5
MAB_3543c	RNA polymerase sigma-E factor	<0.01	4.5
MAB_3233	Hypothetical protein	<0.01	4.5

APPENDIX I

Significantly up-regulated genes (q-value <0.05) in 7C (continued)

Gene	Gene product	q-value	Fold change
MAB_1527	Probable monooxygenase	<0.01	4.4
MAB_1058	Hypothetical protein	<0.01	4.4
MAB_4047c	Sensory transduction protein RegX3	<0.01	4.3
MAB_1357c	Hypothetical protein	<0.01	4.2
MAB_3944	Hypothetical protein	<0.01	4.1
MAB_2799	Hypothetical protein	<0.01	4.0
MAB_2801	Hypothetical protein	<0.01	3.9
MAB_4410c	PEP phosphonomutase and related enzymes	<0.01	3.9
MAB_4714c	Probable fatty-acid-coa ligase FadD	<0.01	3.7
MAB_0485	Putative oxidoreductase	<0.01	3.7
MAB_3776	Hypothetical protein	<0.01	3.6
MAB_2740c	Probable oxidoreductase	<0.01	3.5
MAB_0669	Hypothetical PPE-family protein	<0.01	3.5
MAB_4525	Hypothetical protein	<0.01	3.5
MAB_0661	Putative long chain fatty acid-coA ligase	<0.01	3.5
MAB_4661	Hypothetical protein	<0.01	3.4
MAB_0828	Hypothetical protein	<0.01	3.4
MAB_3402	Hypothetical protein	<0.01	3.3
MAB_0663	Probable cyclic synthetase (peptide synthase)	<0.01	3.2
MAB_3340	Probable sensor histidine kinase	<0.01	3.2
MAB_0671	Hypothetical protein	0.04	3.2
MAB_3055c	Hypothetical protein	<0.01	3.0
MAB_3028	RNA polymerase sigma factor	<0.01	3.0
MAB_1118c	Hypothetical protein	<0.01	3.0
MAB_4170	Hypothetical protein	0.01	3.0
MAB_3778	Hypothetical protein	<0.01	3.0
MAB_4239	Putative pyridoxamine 5'-phosphate oxidase	<0.01	2.9

APPENDIX I

Significantly up-regulated genes (q-value <0.05) in 7C (continued)

Gene	Gene product	q-value	Fold change
MAB_1573c	Hypothetical protein	<0.01	2.9
MAB_1319	Probable 7,8-didemethyl-8-hydroxy-5-deazariboflavin synthase (FO synthase)	<0.01	2.9
MAB_4197	Phosphomethylpyrimidine kinase (ThiD)	<0.01	2.9
MAB_3360c	Hypothetical protein	<0.01	2.8
MAB_0978	Hypothetical protein	<0.01	2.8
MAB_4134	Putative lipase	<0.01	2.8
MAB_4747	Hypothetical protein	<0.01	2.8
MAB_3689	Putative methyltransferase	<0.01	2.7
MAB_3054c	Hypothetical protein	<0.01	2.7
MAB_2595	Putative pyridoxamine 5'-phosphate oxidase	<0.01	2.7
MAB_0829	Putative oxidoreductase	<0.01	2.7
MAB_0660	Hypothetical protein	<0.01	2.7
MAB_4142c	Hypothetical protein	<0.01	2.7
MAB_0667	Hypothetical heavy metal transport/detoxification protein	<0.01	2.6
MAB_3840	Hypothetical protein	<0.01	2.6
MAB_4673c	Putative aminotransferase/cysteine desulfhydrase	<0.01	2.6
MAB_0182c	Hypothetical protein	<0.01	2.6
MAB_3900c	Hypothetical protein	<0.05	2.5
MAB_4437	Probable acyl-CoA dehydrogenase FadE	<0.01	2.5
MAB_0747	Putative phosphate ABC transporter, permease protein	<0.01	2.5
MAB_0670	Hypothetical protein	<0.01	2.5
MAB_3841	Ornithine aminotransferase RocD1	<0.01	2.5
MAB_0664	PE family protein	<0.01	2.5
MAB_1067	Hypothetical protein	<0.01	2.4

APPENDIX I

Significantly up-regulated genes (q-value <0.05) in 7C (continued)

Gene	Gene product	q-value	Fold change
MAB_4671c	Probable ABC transporter, ATP-binding protein	<0.01	2.4
MAB_2802c	Putative ABC-type transporter, permease component	<0.01	2.4
MAB_1412	Hypothetical protein	<0.01	2.4
MAB_0659	Putative dioxygenase	<0.01	2.4
MAB_4672c	Probable ABC transporter, permease protein	<0.01	2.4
MAB_0665	PE family protein	<0.01	2.4
MAB_0048	Probable PE family protein	<0.01	2.4
MAB_3401	Putative monooxygenase	<0.01	2.4
MAB_3525c	adenylyltransferase/sulfurtransferase MoeZ	<0.01	2.3
MAB_3378c	Conserved hypothetical protein (transferase?)	<0.01	2.3
MAB_2229c	Hypothetical PE family protein	<0.01	2.3
MAB_2512	Conserved hypothetical protein (sulfate transporter/antisigma-factor antagonist STAS?)	<0.01	2.3
MAB_1173c	Hypothetical protein	<0.01	2.3
MAB_4940	Thioredoxin reductase (TrxB)	<0.01	2.3
MAB_4665c	Putative short-chain dehydrogenase/reductase	<0.01	2.3
MAB_3951	Hypothetical protein	<0.01	2.3
MAB_1519	Hypothetical protein	<0.01	2.3
MAB_2706c	Putative transporter	<0.01	2.3
MAB_3843	Hypothetical protein	<0.01	2.3
MAB_4265c	Chaperone ClpB	<0.01	2.2
MAB_2270c	Putative peroxidase	<0.01	2.2

APPENDIX I

Significantly up-regulated genes (q-value <0.05) in 7C (continued)

Gene	Gene product	q-value	Fold change
MAB_3400	NADP-dependent alcohol dehydrogenase C	<0.01	2.2
MAB_0046	Probable PE family protein	<0.01	2.2
MAB_4402	Heat shock protein Hsp20	<0.01	2.2
MAB_3024	Hypothetical protein	<0.01	2.2
MAB_2049c	Probable ferredoxin	<0.01	2.2
MAB_2714c	Hypothetical protein	<0.01	2.2
MAB_0668c	Probable O-methyltransferase OmT	<0.01	2.2
MAB_4623c	5-methyltetrahydropteroyltriglutamate--homocysteine S-methyltransferase	<0.01	2.1
MAB_4668c	AMP-dependent synthetase and ligase	<0.01	2.1
MAB_0751c	Phosphate transport system protein PhoU	<0.01	2.1
MAB_1957	Hypothetical protein	<0.01	2.1
MAB_4675c	Acyl-protein synthetase	<0.01	2.1
MAB_4240c	Putative membrane protein, MmpL	<0.01	2.1
MAB_3017	Conserved hypothetical protein (endoribonuclease?)	<0.01	2.1
MAB_0013c	Probable arylamine n-acetyl transferase	<0.01	2.1
MAB_1172c	Hypothetical protein	<0.01	2.1
MAB_2507	Putative oxidoreductase	<0.01	2.1
MAB_2204	Conserved hypothetical protein (FxsA cytoplasmic membrane protein?)	<0.01	2.0
MAB_3994c	Hypothetical protein	<0.01	2.0
MAB_2797c	Putative riboflavin synthase alpha chain	<0.01	2.0
MAB_3839c	Putative transcriptional regulator, AsnC family	<0.01	2.0
MAB_4196	Thiamine biosynthesis protein ThiC	<0.01	2.0
MAB_1464	Hypothetical protein	<0.01	2.0

APPENDIX I

Significantly up-regulated genes (q-value <0.05) in 7C (continued)

Gene	Gene product	q-value	Fold change
MAB_2232c	Putative FtsK/SpoIIIE family protein	<0.01	2.0
MAB_4941	Thioredoxin (Trx)	<0.01	2.0
MAB_3952	Possible O-succinylbenzoic acid--CoA ligase MenE	<0.01	2.0
MAB_1932c	Probable chaperone protein HchA (Hsp31)	<0.01	2.0
MAB_2906c	Hypothetical protein	<0.01	2.0
MAB_4662c	Hypothetical protein	<0.01	2.0
MAB_2698c	Putative RarD protein	0.02	2.0
MAB_3486	Probable acyl-CoA dehydrogenase	<0.01	2.0
MAB_1424c	Putative transcriptional regulator, TetR family	<0.01	2.0
MAB_2530c	Catalase CatB	<0.01	1.9
MAB_3033	Hypothetical protein	<0.01	1.9
MAB_2095	Metal-dependent phosphohydrolase, HD subdomain	<0.01	1.9
MAB_1479	Possible thiamineS	<0.01	1.9
MAB_2233c	Hypothetical protein	0.02	1.9
MAB_4494c	Hypothetical protein	<0.01	1.9
MAB_1567	Probable HNH endonuclease precursor	<0.01	1.9
MAB_1291	Hypothetical protein	<0.01	1.9
MAB_3725c	Hypothetical protein	<0.01	1.9
MAB_0145c	Probable transcriptional regulator, TetR family	<0.01	1.9
MAB_0049	ESAT-6-like protein	<0.01	1.9
MAB_2632	Probable ATP-binding protein ABC transporter CydD	<0.01	1.9
MAB_3842	Probable cationic amino acid transport integral membrane protein	<0.01	1.9
MAB_1395	Probable multidrug resistance transporter, Bcr/CflA family	<0.01	1.9

APPENDIX I

Significantly up-regulated genes (q-value <0.05) in 7C (continued)

Gene	Gene product	q-value	Fold change
MAB_1363	Hypothetical protein	<0.01	1.9
MAB_0370	Hypothetical protein	<0.01	1.9
MAB_3450c	Probable phosphoglucomutase PgmA	<0.01	1.9
MAB_2228c	ESAT-6-like protein esxH	<0.01	1.9
MAB_4326c	Hypothetical protein	<0.01	1.9
MAB_0742c	Probable thioredoxin ThiX	0.03	1.8
MAB_4015c	Hypothetical protein	<0.01	1.8
MAB_3147c	Probable conserved polyketide synthase associated protein	<0.01	1.8
MAB_1171c	Hypothetical protein	<0.01	1.8
MAB_0666	ESAT-6-like protein (10 kDa antigen)	<0.01	1.8
MAB_1812c	Hypothetical protein	<0.01	1.8
MAB_2508	Hypothetical protein	<0.01	1.8
MAB_2248	Probable peptide synthetase MbtE	<0.01	1.8
MAB_2762	Putative OxpP cycle protein OpcA	<0.01	1.8
MAB_2230c	Hypothetical PPE family protein	<0.01	1.8
MAB_4229c	Hypothetical protein	<0.01	1.8
MAB_1601c	Putative monooxygenase	<0.01	1.8
MAB_2763	6-phosphogluconolactonase	<0.01	1.8
MAB_4307	Possible arylsulfatase AtsA	<0.01	1.8
MAB_1931c	Hypothetical protein	<0.01	1.8
MAB_1324	Putative monooxygenase	<0.05	1.8
MAB_2699c	Probable pseudouridine synthase RluD	<0.01	1.8
MAB_1427c	Putative cytochrome P450	<0.01	1.8
MAB_4216c	Thiazole biosynthesis protein ThiG	<0.01	1.8
MAB_2140	NADH-quinone oxidoreductase, G subunit NuoG	<0.01	1.8
MAB_2446c	Putative pirin-like protein	<0.01	1.8

APPENDIX I

Significantly up-regulated genes (q-value <0.05) in 7C (continued)

Gene	Gene product	q-value	Fold change
MAB_2234c	Conserved hypothetical protein (AAA ATPase?)	0.03	1.8
MAB_2715c	Hypothetical protein	<0.01	1.8
MAB_2157	Probable acyl-[acyl-carrier protein] desaturase	<0.01	1.8
MAB_2961	Putative menaquinone biosynthesis methyltransferase	0.02	1.8
MAB_0047	Probable PPE family protein	<0.01	1.8
MAB_2269c	Putative transcriptional regulator, MerR family	<0.01	1.8
MAB_1396	Probable drug resistance transporter, EmrB/QacA subfamily	<0.01	1.8
MAB_0126c	Possible bacterioferritin BfrB	<0.01	1.8
MAB_1194	Putative lipase LipU	<0.01	1.7
MAB_2268c	Hypothetical protein	<0.01	1.7
MAB_2760	Probable transaldolase	<0.01	1.7
MAB_1480	Probable cysteine synthase	<0.01	1.7
MAB_0693	Probable glutathione peroxidase	<0.01	1.7
MAB_4739	Probable FAD dependent oxidoreductase	<0.01	1.7
MAB_2480	Hypothetical protein	<0.01	1.7
MAB_0371	Hypothetical protein	<0.01	1.7
MAB_1969c	Probable cytochrome c oxidase subunit III	<0.01	1.7
MAB_2225c	Probable peptidase	<0.01	1.7
MAB_2889	Hypothetical protein	<0.01	1.7
MAB_3035	Conserved hypothetical protein (phenazine biosynthesis?)	<0.01	1.7
MAB_4607c	Hypothetical protein	<0.01	1.7
MAB_0967	Hypothetical protein	<0.01	1.7

APPENDIX I

Significantly up-regulated genes (q-value <0.05) in 7C (continued)

Gene	Gene product	q-value	Fold change
MAB_1478	Hypothetical protein	<0.01	1.7
MAB_2134	NADH-quinone oxidoreductase, A subunit NuoA	<0.01	1.7
MAB_4456	Putative cytochrome P450	<0.01	1.7
MAB_0372	Hypothetical protein	<0.01	1.7
MAB_1481	Hypothetical protein	0.01	1.7
MAB_2249	Probable lysine-N-oxygenase MbtG	<0.01	1.7
MAB_4634c	Putative TetR-family transcriptional regulator	<0.01	1.7
MAB_3029	Iron-dependent repressor IdeR	<0.01	1.7
MAB_2226c	Hypothetical protein	<0.01	1.7
MAB_0947c	Putative luciferase	<0.01	1.7
MAB_1838	Endonuclease	<0.01	1.7
MAB_3068c	Possible transcriptional regulatory protein	<0.01	1.7
MAB_2513c	Anti-sigma factor RsbW	<0.01	1.7
MAB_4393	Hypothetical protein	<0.01	1.7
MAB_0120	Probable peptide methionine sulfoxide reductase	<0.01	1.7
MAB_1888	Conserved hypothetical protein (thioesterase?)	<0.01	1.7
MAB_0867c	Putative transferase	<0.01	1.7
MAB_2651c	Probable oxidoreductase	<0.01	1.7
MAB_4730	Putative LysR-family transcriptional regulator	<0.01	1.7
MAB_3556	Putative hydrolase, alpha/beta fold	<0.01	1.7
MAB_2227c	Hypothetical protein	<0.01	1.7
MAB_2430c	Hypothetical protein	<0.01	1.6
MAB_1107c	Hypothetical protein	0.01	1.6
MAB_2761	Glucose-6-phosphate 1-dehydrogenase	<0.01	1.6

APPENDIX I

Significantly up-regulated genes (q-value <0.05) in 7C (continued)

Gene	Gene product	q-value	Fold change
MAB_3014	Putative FAD-dependent pyridine nucleotide-disulphide oxidoreductase, similar to mercuric reductases protein	<0.01	1.6
MAB_2224c	Hypothetical protein	<0.01	1.6
MAB_2271c	Hypothetical protein	<0.01	1.6
MAB_3583c	Hypothetical protein	<0.01	1.6
MAB_1482	Probable glutamate racemase MurI	0.03	1.6
MAB_4050c	Probable monooxygenase	<0.01	1.6
MAB_2187	Sec-independent protein translocase protein TatA/E	<0.01	1.6
MAB_0755c	Hypothetical protein	<0.01	1.6
MAB_2674c	Probable quinolinate synthetase complex, A subunit (NadA)	0.04	1.6
MAB_2847c	Hypothetical protein	0.01	1.6
MAB_4162c	Putative 2-nitropropane dioxygenase	<0.01	1.6
MAB_0373	Probable aminotransferase	<0.01	1.6
MAB_3468	Putative transcriptional regulator, MerR family	0.03	1.6
MAB_2697c	Hypothetical protein	<0.01	1.6
MAB_2435	Molybdenum ABC transporter ModC, ATP-binding protein	<0.01	1.6
MAB_3558	Hypothetical protein	<0.05	1.6
MAB_1854c	Glycogen phosphorylase	<0.01	1.6
MAB_2852c	Hypothetical protein	<0.01	1.6
MAB_1724c	Hypothetical protein	<0.01	1.6
MAB_2107	Hypothetical protein	<0.01	1.6
MAB_3245	Hypothetical protein	<0.01	1.6
MAB_3512	Putative glutaredoxin-like protein	0.04	1.6

APPENDIX I

Significantly up-regulated genes (q-value <0.05) in 7C (continued)

Gene	Gene product	q-value	Fold change
MAB_2237c	Probable FAD-dependent monooxygenase	0.02	1.6
MAB_1429	Putative permease of the major facilitator superfamily	<0.01	1.6
MAB_2244	Fumarate reductase/succinate dehydrogenase flavoprotein-like FrdA	0.04	1.6
MAB_2581	Putative two component transcriptional regulatory protein	<0.01	1.6
MAB_0543	Conserved hypothetical protein (Bvg accessory factor?)	<0.01	1.6
MAB_2120c	Putative polyketide synthase MbtC	<0.01	1.6
MAB_2179	Beta-lactamase/esterase	<0.01	1.6
MAB_2415c	Conserved hypothetical protein (penicillinase repressor?)	<0.01	1.6
MAB_3598c	Putative alkane-1-monooxygenase AlkB (fatty acid omega-hydroxylase)	<0.01	1.6
MAB_3429	Putative integral membrane protein	<0.01	1.6
MAB_1332	Probable fatty-acid-CoA ligase FadD	<0.01	1.6
MAB_1394c	Hypothetical protein	<0.01	1.6
MAB_3891c	Probable transcriptional regulator, LuxR family	<0.01	1.6
MAB_0291	CdaR family transcriptional regulator	<0.01	1.6
MAB_3731c	60 kDa chaperonin 1 (GroEL protein 1)	<0.01	1.6
MAB_1330	1-pyrroline-5-carboxylate dehydrogenase	<0.01	1.5
MAB_0246	Hypothetical protein	<0.05	1.5
MAB_3082	Hypothetical protein	0.04	1.5
MAB_4218c	Possible thiamine biosynthesis oxidoreductase ThiO	<0.01	1.5

APPENDIX I

Significantly up-regulated genes (q-value <0.05) in 7C (continued)

Gene	Gene product	q-value	Fold change
MAB_1426	Putative cytochrome P450	<0.01	1.5
MAB_0929	Hypothetical protein	<0.01	1.5
MAB_2614	Hypothetical monooxygenase, FAD-binding	<0.01	1.5
MAB_1178c	Hypothetical protein	<0.01	1.5
MAB_0650	60 kDa chaperonin 2 (Protein Cpn60 2) (GroEL)	<0.01	1.5
MAB_3428c	Probable RNA polymerase sigma-C factor	<0.01	1.5
MAB_2901c	Conserved hypothetical protein (thioesterase?)	<0.01	1.5
MAB_2704	Hypothetical protein	<0.01	1.5
MAB_4434c	Putative iron/ascorbate dependent oxidoreductase	0.04	1.5
MAB_3827c	Ethanolamine ammonia-lyase, small subunit	0.02	1.5
MAB_0587	Probable acetamidase/formamidase	<0.01	1.5
MAB_4720c	Hypothetical protein	<0.01	1.5
MAB_3837	Probable transcriptional regulatory protein TetR	<0.01	1.5
MAB_4205	Hypothetical protein	0.02	1.5
MAB_4271c	Chaperone protein DnaJ	<0.01	1.5
MAB_2047c	Probable ferredoxin reductase	<0.01	1.5
MAB_3487	Probable acyl-CoA dehydrogenase	<0.01	1.5
MAB_3034	Probable hydrolase	<0.01	1.5
MAB_1476	Hypothetical protein	0.04	1.5
MAB_1368	Hypothetical protein	<0.01	1.5
MAB_3732c	10 kDa chaperonin (GroES)	<0.01	1.5
MAB_0068	Putative transcriptional regulator, GntR family	<0.01	1.5

APPENDIX I

Significantly up-regulated genes (q-value <0.05) in 7C (continued)

Gene	Gene product	q-value	Fold change
MAB_0993c	Putative transcriptional regulator, LysR family	<0.01	1.5
MAB_4785	Hypothetical protein	0.04	1.5
MAB_1233c	Hypothetical protein	0.02	1.5
MAB_4290c	Hypothetical protein	<0.01	1.5
MAB_1151	Hypothetical protein	<0.01	1.5
MAB_1477	Conserved hypothetical protein (peptidase?)	<0.01	1.5
MAB_2577	Putative TetR-family transcriptional regulator	<0.01	1.5
MAB_4944c	Putative cytochrome P450	<0.05	1.5
MAB_4415	Probable amidase	<0.01	1.5
MAB_1561	Probable NAD-dependent glutamate dehydrogenase	<0.01	1.5
MAB_3318	Putative regulatory protein	<0.01	1.5
MAB_3260c	Hypothetical protein	<0.01	1.5
MAB_2467	Ubiquinol-cytochrome c reductase cytochrome b subunit	<0.01	1.5
MAB_4224	Probable serine/threonine-protein kinase	0.02	1.5
MAB_4441	Putative oxidoreductase	<0.01	1.5
MAB_4919	Putative transcriptional regulator, AsnC family	0.02	1.5
MAB_0183c	Putative cation transporter	<0.01	1.5
MAB_3719c	Putative cholesterol oxidase ChoD	<0.01	1.5
MAB_2048c	Probable cytochrome P450	<0.01	1.5
MAB_0274c	Hypothetical protein	<0.01	1.5
MAB_1593c	Probable formate dehydrogenase, A chain	<0.01	1.5
MAB_2707	Putative transcriptional regulator	<0.05	1.5

APPENDIX I

Significantly up-regulated genes (q-value <0.05) in 7C (continued)

Gene	Gene product	q-value	Fold change
MAB_0464	Hypothetical protein	<0.01	1.5
MAB_0568	Putative CarD-like transcriptional regulator	<0.01	1.5
MAB_1176c	Hypothetical protein	<0.01	1.5
MAB_1762	Hypothetical protein	0.04	1.5
MAB_1556	Hypothetical protein	<0.01	1.5
MAB_1292c	Probable O-methyltransferase OMT	<0.01	1.5
MAB_1871	Hypothetical protein	<0.01	1.5
MAB_0800	Hypothetical protein	<0.01	1.5
MAB_0541	Probable pantoate--beta-alanine ligase (PanC)	<0.01	1.5
MAB_0121	Rhodanese-like protein	<0.05	1.5
MAB_2445	Putative transcriptional regulator, AraC family	<0.01	1.5
MAB_3844c	Putative short-chain dehydrogenase/reductase	<0.01	1.5
MAB_3857c	Probable enoyl-coa hydratase/isomerase	<0.01	1.5
MAB_1944c	Hypothetical protein	<0.01	1.5
MAB_1668	PhoH-like protein	<0.01	1.5
MAB_4136c	Putative aldehyde dehydrogenase	<0.01	1.5
MAB_3627c	Possible oxidoreductase	<0.01	1.5
MAB_4052c	Putative lipase/esterase	<0.01	1.5
MAB_0542	Probable aspartate 1-decarboxylase precursor	<0.01	1.5
MAB_0213c	Hypothetical protein	<0.01	1.4
MAB_3838c	Putative ferredoxin reductase	<0.01	1.4
MAB_4622c	Hypothetical protein	<0.01	1.4
MAB_4076	Putative nitrilase/cyanide hydratase	<0.01	1.4

APPENDIX I

Significantly up-regulated genes (q-value <0.05) in 7C (continued)

Gene	Gene product	q-value	Fold change
MAB_4724	Sodium/calcium exchanger family protein	<0.01	1.4
MAB_3780	DTDP-4-dehydrorhamnose 3,5-epimerase RmlC	<0.01	1.4
MAB_4303	Alpha galactosidase precursor	<0.01	1.4
MAB_1032c	Conserved hypothetical protein (Ku70/Ku80 beta-barrel domain protein?)	<0.01	1.4
MAB_4419	Tartrate dehydrogenase	0.03	1.4
MAB_0601	Hypothetical protein	<0.01	1.4
MAB_0418	Probable endonuclease III protein	<0.01	1.4
MAB_4411c	Putative transcriptional regulator, GntR family	0.02	1.4
MAB_1177	Hypothetical protein	<0.01	1.4
MAB_3335c	6-phosphofructokinase PfkA	<0.01	1.4
MAB_1489	Probable acyl-CoA ligase FadD	<0.01	1.4
MAB_1239	Probable PhoH-like protein PhoH2 (phosphate starvation-inducible protein PsiH)	<0.01	1.4
MAB_2489	Hypothetical protein	<0.01	1.4
MAB_3741c	Hypothetical protein	<0.01	1.4
MAB_4137c	Hypothetical protein	<0.01	1.4
MAB_4072c	Hypothetical protein	0.03	1.4
MAB_4606c	Hypothetical protein	0.03	1.4
MAB_3941c	Hypothetical protein	<0.01	1.4
MAB_1485	HAM1 protein homolog (NTPase)	<0.01	1.4
MAB_3977c	Conserved hypothetical protein (phosphoglycerate mutase?)	<0.01	1.4
MAB_4799c	Conserved hypothetical protein (methyltransferase?)	0.02	1.4

APPENDIX I

Significantly up-regulated genes (q-value <0.05) in 7C (continued)

Gene	Gene product	q-value	Fold change
MAB_4806c	Hypothetical protein	<0.01	1.4
MAB_0540	Hypothetical protein	<0.01	1.4
MAB_4548	Probable O-methyltransferase	<0.01	1.4
MAB_4629	Hypothetical protein	0.04	1.4
MAB_3763	Probable cutinase cut2 precursor	0.02	1.4
MAB_2793c	Putative transcriptional regulator	<0.01	1.4
MAB_2860c	Hypothetical protein	<0.01	1.4
MAB_4169	Possible lipid carrier protein or keto acyl-COA thiolase	0.02	1.4
MAB_4222	Hypothetical protein	<0.05	1.4
MAB_2122	Putative peptide synthetase MbtE	0.02	1.4
MAB_0750	Putative oxidoreductase	<0.01	1.4
MAB_1439c	Hypothetical protein	<0.01	1.4
MAB_2984c	Putative chlorite dismutase	0.02	1.4
MAB_1257	4-hydroxy-3-methylbut-2-enyl diphosphate reductase	<0.01	1.4
MAB_2431c	Possible luciferase-like oxidoreductase	<0.01	1.4
MAB_1475	Probable ATP-dependent Clp protease adaptor protein ClpS	<0.01	1.4
MAB_0745	Putative acetyltransferase	<0.01	1.4
MAB_2091	Hypothetical protein similar to Patatin	0.01	1.4
MAB_1209	Probable short-chain Z-isoprenyl diphosphate synthetase	<0.01	1.4
MAB_3761c	Hypothetical protein	<0.01	1.4
MAB_1589	Putative transcriptional regulator, TetR family	<0.01	1.4
MAB_1842c	Probable catalase	<0.01	1.4
MAB_2133	Probable response regulator	<0.01	1.4

APPENDIX I

Significantly up-regulated genes (q-value <0.05) in 7C (continued)

Gene	Gene product	q-value	Fold change
MAB_3007c	Probable inositol-1-monophosphatase SuhB	0.02	1.4
MAB_2473c	Hypothetical protein	<0.01	1.4
MAB_3133c	Putative flavohemoprotein	<0.01	1.4
MAB_3148c	Probable polyketide synthase Pks5	0.02	1.4
MAB_0730c	Conserved hypothetical protein (glycine cleavage T-protein aminomethyl transferase?)	<0.01	1.4
MAB_2746c	Probable cysteine desulfurase/aminotransferase	<0.01	1.4
MAB_3174	Hypothetical protein	<0.01	1.4
MAB_4086	Possible TetR-family transcriptional regulator	<0.01	1.4
MAB_3368c	Putative transcriptional regulator, AsnC family	0.02	1.4
MAB_0027c	Hypothetical protein	<0.01	1.4
MAB_1943c	Probable lipoyltransferase LipB	0.04	1.4
MAB_2962	Probable fatty-acid-CoA ligase FadD	<0.01	1.4
MAB_3032	Probable soluble pyridine nucleotide transhydrogenase	<0.01	1.4
MAB_2747c	ABC transporter ATP-binding protein	<0.01	1.4
MAB_0257c	Hypothetical protein	<0.01	1.4
MAB_0104	Probable enoyl-CoA hydratase/isomerase	0.04	1.4
MAB_4230c	F420-dependent glucose-6-phosphate dehydrogenase	<0.01	1.4
MAB_2429c	Probable NADH dehydrogenase (NDH)	<0.01	1.4
MAB_3081	Short-chain dehydrogenase/reductase	<0.01	1.4
MAB_4175c	D-tyrosyl-tRNA(Tyr) deacylase	<0.01	1.4
MAB_3834c	Possible L-lactate dehydrogenase (cytochrome) LldD1	<0.01	1.4

APPENDIX I

Significantly up-regulated genes (q-value <0.05) in 7C (continued)

Gene	Gene product	q-value	Fold change
MAB_2202	Hypothetical protein	0.02	1.4
MAB_4328c	Hypothetical protein	0.03	1.4
MAB_3902c	Hypothetical protein	<0.01	1.4
MAB_3646c	Probable aminotransferase class-III	0.02	1.4
MAB_0703c	Hypothetical protein	0.02	1.4
MAB_3937	Hypothetical protein	<0.01	1.4
MAB_2245	Putative anthranilate synthase component I TrpE2/ Salicylate synthase MbtI	<0.01	1.4
MAB_1193c	Conserved hypothetical protein (lipolytic enzyme G-D-S-L?)	<0.01	1.4
MAB_1927	Putative sensor-type histidine kinase PrrB	0.01	1.4
MAB_4417c	Probable aminotransferase	<0.01	1.4
MAB_2859c	Transglutaminase family protein	<0.01	1.4
MAB_0452c	Hypothetical protein	<0.01	1.4
MAB_2757	Probable quinone reductase Qor	<0.01	1.4
MAB_0942	Putative transcriptional regulator, AraC family	0.03	1.4
MAB_1078	Putative serine protease	0.03	1.4
MAB_2119c	Putative polyketide synthase MbtD	<0.01	1.4
MAB_4712	Putative transcriptional regulator, AraC	<0.01	1.3
MAB_3701c	Putative AraC-family regulatory protein	0.04	1.3
MAB_2123	Putative peptide synthetase MbtF	<0.05	1.3
MAB_1229	Hypothetical protein	<0.01	1.3
MAB_2719c	Hypothetical protein	<0.01	1.3
MAB_0831	Hypothetical protein	<0.01	1.3
MAB_2727c	Hypothetical invasion protein Inv2	<0.01	1.3
MAB_1845c	Hypothetical protein	<0.05	1.3
MAB_3582	Putative transcriptional regulator, GntR family	0.03	1.3

APPENDIX I

Significantly up-regulated genes (q-value <0.05) in 7C (continued)

Gene	Gene product	q-value	Fold change
MAB_3388c	Probable phosphoserine phosphatase (SerB2)	<0.01	1.3
MAB_0118c	Probable superoxide dismutase (Mn)	<0.01	1.3
MAB_4266c	Hypothetical protein	<0.01	1.3
MAB_4284c	Hypothetical protein	<0.01	1.3
MAB_4947	Beta-lactamase-like protein	0.01	1.3
MAB_1962	Probable cytochrome c oxidase polypeptide 4	<0.01	1.3
MAB_2925c	Xanthine dehydrogenase family protein	0.03	1.3
MAB_4184c	Superoxide dismutase [Cu-Zn] precursor	<0.01	1.3
MAB_3835c	Probable coenzyme PQQ synthesis protein E PqqE	<0.01	1.3
MAB_2148	Putative linoleoyl-CoA desaturase	<0.01	1.3
MAB_3441c	NAD-dependent deacetylase (regulatory protein Sir2 homolog)	0.02	1.3
MAB_0930	Putative ferredoxin/ferredoxin--NADP reductase	<0.01	1.3
MAB_1152	Hypothetical protein	0.04	1.3
MAB_3957	Superoxide dismutase SodM	0.01	1.3
MAB_2471c	Ferric uptake regulation protein FurA	0.01	1.3
MAB_0868c	Hypothetical protein	0.01	1.3
MAB_2805c	Hypothetical protein	<0.01	1.3
MAB_4677c	Hypothetical protein	0.01	1.3
MAB_2586	Putative transcription antitermination regulator	0.02	1.3
MAB_2928	Probable amidohydrolase	0.04	1.3
MAB_1030	Hypothetical protein	0.01	1.3
MAB_4231	Beta-lactamase-like protein	<0.01	1.3

APPENDIX I

Significantly up-regulated genes (q-value <0.05) in 7C (continued)

Gene	Gene product	q-value	Fold change
MAB_4585c	Hypothetical protein	0.01	1.3
MAB_4273c	Chaperone protein DnaK (Hsp 70)	0.03	1.3
MAB_1432	Hypothetical protein	0.01	1.3
MAB_1371	Hypothetical protein	<0.01	1.3
MAB_2885	Putative transcriptional regulator, TetR-family	0.01	1.3
MAB_2124	Putative phenyloxazoline synthase MbtB	<0.01	1.3
MAB_2661c	Putative nitroreductase	0.02	1.3
MAB_1725c	Acyltransferase	<0.01	1.3
MAB_1996	Hypothetical protein	<0.01	1.3
MAB_0672c	Hypothetical protein	0.02	1.3
MAB_2960	Hypothetical protein	<0.01	1.3
MAB_1318c	Probable catechol-o-methyltransferase	<0.01	1.3
MAB_3679c	Hypothetical protein	0.02	1.3
MAB_2630	Probable integral membrane cytochrome D ubiquinol oxidase (Subunit I) CydA	0.01	1.3
MAB_1153	Hypothetical protein	0.01	1.3
MAB_1875c	Putative hydrolase (alpha/beta fold)	<0.01	1.3
MAB_2247c	2,3-dihydroxybenzoate-AMP ligase	0.03	1.3
MAB_3670c	Adenosine deaminase	0.04	1.3
MAB_4046c	Putative transcriptional regulator, TetR family	0.02	1.3
MAB_3647c	Probable transcriptional regulatory protein	0.02	1.3
MAB_2023c	Putative LacI-family transcriptional regulator	0.04	1.3
MAB_4280c	Hypothetical protein	0.02	1.3
MAB_2162	Putative AAA-family ATPase	0.02	1.3
MAB_1069c	Probable enoyl-CoA hydratase/isomerase	0.02	1.3

APPENDIX I

Significantly up-regulated genes (q-value <0.05) in 7C (continued)

Gene	Gene product	q-value	Fold change
MAB_3156c	Probable Cob(I)alamin adenosyltransferase (CobO)	0.02	1.3
MAB_4528c	Hypothetical protein	0.02	1.3
MAB_0407c	Conserved hypothetical protein (metallophospho esterase?)	<0.01	1.3
MAB_0739c	Hypothetical protein	<0.01	1.3
MAB_1469	Probable glycogen phosphorylase GlgP	0.01	1.3
MAB_1625	Hypothetical protein	0.02	1.3
MAB_0069	Major facilitator family transporter	<0.01	1.3
MAB_3027	Hypothetical protein	0.02	1.3
MAB_3289	Hypothetical protein	0.02	1.3
MAB_2477c	Probable monooxygenase	0.02	1.3
MAB_1852c	Hypothetical isochorismatase hydrolase	0.02	1.3
MAB_3655c	Probable glycerol-3-phosphate dehydrogenase	0.02	1.3
MAB_4100c	MbtH-like protein	0.04	1.3
MAB_0933	Probable pyridoxamine 5'-phosphate oxidase PdxH	0.03	1.3
MAB_4109c	Putative methyltransferase	0.03	1.3
MAB_3501	Hypothetical protein	0.01	1.3
MAB_4443	Probable 3-oxoacyl-[acyl-carrier protein] reductase	0.01	1.3
MAB_2212	Probable polyketide synthase	<0.05	1.3
MAB_1473c	Hypothetical protein	0.02	1.3
MAB_1146	Hypothetical protein	0.01	1.3
MAB_3206c	Putative transcriptional regulator, TetR	0.03	1.3
MAB_3740c	Probable glutamate decarboxylase GadB	0.02	1.3
MAB_2657c	Hypothetical protein	<0.05	1.3

APPENDIX I

Significantly up-regulated genes (q-value <0.05) in 7C (continued)

Gene	Gene product	q-value	Fold change
MAB_4444	Conserved hypothetical protein (MaoC-like dehydratase)	0.02	1.3
MAB_3633	Maf-like protein	0.02	1.3
MAB_3970c	Hypothetical protein	0.04	1.3
MAB_1719	Hypothetical protein	0.02	1.3
MAB_3168c	Conserved hypothetical protein (acetyltransferase?)	0.02	1.3
MAB_1181c	Putative lipoprotein LpqV precursor	0.04	1.3
MAB_2498c	Hypothetical protein	0.02	1.3
MAB_3243	Soluble secreted antigen MPT53 precursor	0.02	1.3
MAB_1393c	Probable 2-oxoglutarate dehydrogenase SucA	<0.05	1.3
MAB_0152	Putative ankyrin-like protein	0.04	1.3
MAB_3779	DTDP-glucose 4,6-dehydratase RmlB	0.03	1.3
MAB_1571c	Probable aminopeptidase	0.04	1.2
MAB_2834c	Conserved hypothetical protein (methyltransferase?)	0.03	1.2
MAB_3066c	Hypothetical protein	0.04	1.2
MAB_2748c	Hypothetical protein	0.03	1.2
MAB_1925	Hypothetical protein	0.03	1.2
MAB_0600	Probable acetyl-CoA acetyltransferase FadA	0.04	1.2
MAB_0103	Probable monooxygenase EthA	0.03	1.2
MAB_3789c	Possible protease IV SppA (endopeptidase IV)	0.04	1.2
MAB_1040	Hypothetical protein	0.04	1.2
MAB_4630	Hypothetical protein	0.04	1.2
MAB_1051c	Probable glucose-6-phosphate isomerase (PGI)	0.04	1.2

APPENDIX I

Significantly up-regulated genes (q-value <0.05) in 7C (continued)

Gene	Gene product	q-value	Fold change
MAB_1462c	Conserved hypothetical protein (Glyoxalase/ Bleomycin resistance protein?)	<0.05	1.2
MAB_3722	Hypothetical protein	<0.05	1.2
MAB_1981	Hypothetical protein	<0.05	1.2
MAB_2749c	Putative FeS assembly protein SufB	<0.05	1.2
MAB_0968c	Luciferase-like hypothetical protein	<0.05	1.2
MAB_4423	Putative succinate dehydrogenase, iron-sulfur subunit	<0.05	1.2

APPENDIX J

Significantly down-regulated genes (q-value <0.05) in 7C

Gene	Gene product	q-value	Fold change
MAB_4791c	Hypothetical protein	<0.01	∞
MAB_4803	Hypothetical protein	<0.01	∞
MAB_2903	Hypothetical protein	0.02	-19.3
MAB_4095c	Isocitrate lyase (AceA)	<0.01	-7.2
MAB_3716	Hypothetical protein	<0.01	-6.6
MAB_1263	Hypothetical protein	<0.01	-6.5
MAB_4706c	Hypothetical protein	<0.01	-6.1
MAB_4131	Hypothetical protein	<0.01	-6.1
MAB_3065c	Hypothetical protein	<0.01	-4.9
MAB_1261	Hypothetical protein	<0.01	-4.7
MAB_1732	Hypothetical protein	<0.01	-4.3
MAB_0909	Putative phenylacetic acid degradation protein PaaD/phenylacetate-CoA oxygenase, PaaJ subunit	<0.01	-4.2
MAB_0906	Putative phenylacetic acid degradation protein PaaA/phenylacetate-CoA oxygenase, PaaG subunit	<0.01	-4.1
MAB_1264	Hypothetical protein	<0.01	-4.1
MAB_0911	Putative phenylacetate-CoA ligase	<0.01	-4.1
MAB_0910	Putative phenylacetic acid degradation protein PaaE/phenylacetate-CoA oxygenase/reductase, PaaK subunit	<0.01	-3.9
MAB_2693	Hypothetical protein	0.04	-3.9
MAB_1262	Hypothetical protein	<0.01	-3.9
MAB_0908	Putative phenylacetic acid degradation protein PaaC/phenylacetate-CoA oxygenase, PaaI subunit	<0.01	-3.9

∞ indicates that the gene was expressed in *M. abscessus* ATCC 19977 but not in 7C.

APPENDIX J

Significantly down-regulated genes (q-value <0.05) in 7C (continued)

Gene	Gene product	q-value	Fold change
MAB_0907	Putative phenylacetic acid degradation protein PaaB/phenylacetate-CoA oxygenase, PaaH subunit	<0.01	-3.8
MAB_4579c	Probable NAD(P) transhydrogenase, alpha1 subunit PntAA	<0.01	-3.6
MAB_1826	Hypothetical protein	<0.01	-3.6
MAB_0904	Putative 3-hydroxyacyl-CoA dehydrogenase	<0.01	-3.5
MAB_0900c	Putative phenylacetic acid degradation protein PaaN	<0.01	-3.4
MAB_0905	Putative enoyl-CoA hydratase/isomerase	<0.01	-3.4
MAB_4578c	Probable NAD(P) transhydrogenase, alpha2 subunit PntAB	0.03	-3.4
MAB_3064	Hypothetical protein	<0.01	-3.4
MAB_4094c	Probable 3-hydroxybutyryl-CoA dehydrogenase	<0.01	-3.3
MAB_4084c	Possible transcriptional regulator	<0.01	-3.2
MAB_0902	Probable beta-ketoadipyl-CoA thiolase	<0.01	-3.1
MAB_0903	Putative enoyl-CoA hydratase/isomerase	<0.01	-3.0
MAB_0899c	Possible ethyl tert-butyl ether degradation protein EthD	<0.01	-3.0
MAB_0897c	Probable pyruvate dehydrogenase E1 component, alpha subunit	<0.01	-3.0

∞ indicates that the gene was expressed in *M. abscessus* ATCC 19977 but not in 7C.

APPENDIX J

Significantly down-regulated genes (q-value <0.05) in 7C (continued)

Gene	Gene product	q-value	Fold change
MAB_0895c	Putative dihydrolipoamide s-acetyltransferase component of pyruvate dehydrogenase complex E2	<0.01	-2.9
MAB_3715c	Putative two-component system sensor kinase	<0.01	-2.9
MAB_4371	Putative alcohol dehydrogenase	0.04	-2.8
MAB_0335	Probable cobalamin synthesis protein	<0.01	-2.8
MAB_0894c	Putative dihydrolipoamide dehydrogenase (LpdA)	<0.01	-2.8
MAB_2717c	Hypothetical protein	<0.01	-2.7
MAB_0896c	Putative pyruvate dehydrogenase E1 component, beta subunit	<0.01	-2.7
MAB_2495	Probable acyl-CoA dehydrogenase	<0.01	-2.6
MAB_0912	Hypothetical protein	<0.01	-2.6
MAB_4577c	Probable NAD(P) transhydrogenase, beta subunit PntB	<0.01	-2.5
MAB_4463	Hypothetical protein	<0.01	-2.5
MAB_4509c	Hypothetical protein	<0.01	-2.4
MAB_0893	4-hydroxyphenylpyruvate dioxygenase	<0.01	-2.4
MAB_0898	Probable transcriptional regulator, AsnC family	<0.01	-2.3
MAB_1748	Hypothetical protein	<0.01	-2.3
MAB_0717	Probable dehydrogenase/reductase	<0.01	-2.2
MAB_4083c	Heparin-binding hemagglutinin (adhesin)	<0.01	-2.2
MAB_2379	Hypothetical lipoprotein LpqH precursor	<0.01	-2.2

∞ indicates that the gene was expressed in *M. abscessus* ATCC 19977 but not in 7C.

APPENDIX J

Significantly down-regulated genes (q-value <0.05) in 7C (continued)

Gene	Gene product	q-value	Fold change
MAB_4773	Probable heme oxygenase	<0.01	-2.2
MAB_3714c	Putative two-component system response regulator, LuxR family	<0.01	-2.2
MAB_4558c	Hypothetical protein	<0.01	-2.1
MAB_3107c	Possible lipoprotein LppU	<0.01	-2.1
MAB_1297c	Hypothetical protein	<0.01	-2.1
MAB_0869c	Probable resuscitation-promoting factor RpfA	<0.01	-2.1
MAB_2972	Hypothetical protein	<0.01	-2.0
MAB_1726	Bacteriophage protein	0.01	-2.0
MAB_1742	Hypothetical protein	<0.01	-2.0
rpmB	50S ribosomal protein L28	<0.01	-2.0
MAB_2329c	Hypothetical protein	<0.01	-2.0
MAB_0688c	Hypothetical protein	<0.01	-2.0
rpmF	50S ribosomal protein L32	<0.01	-1.9
rpsT	30S ribosomal protein S20	<0.01	-1.9
MAB_1890c	Hypothetical protein	<0.01	-1.9
MAB_4353	Hypothetical protein	<0.01	-1.9
MAB_3898c	Hypothetical protein	<0.01	-1.9
MAB_3085c	Thymidylate synthase ThyX	<0.01	-1.9
MAB_p21c	Hypothetical protein	<0.01	-1.9
MAB_1053c	Conserved hypothetical protein (chorismate mutase?)	<0.01	-1.9
MAB_0848c	Hypothetical protein	<0.01	-1.9
rplS	50S ribosomal protein L19	<0.01	-1.8
MAB_2273	Putative MFS transporter	<0.01	-1.8
MAB_0809c	Conserved hypothetical PPE family protein	<0.01	-1.8

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APPENDIX J

Significantly down-regulated genes (q-value <0.05) in 7C (continued)

Gene	Gene product	q-value	Fold change
MAB_1608c	Hypothetical protein	<0.01	-1.8
rpsO	30S ribosomal protein S15	<0.01	-1.8
MAB_1265c	Hypothetical protein	0.04	-1.8
MAB_1614	Hypothetical protein	<0.01	-1.8
MAB_1026c	Hypothetical protein	<0.01	-1.8
MAB_1131	16S rRNA (adenine(1518)-N(6)/adenine(1519)-N(6))-dimethyltransferase	<0.01	-1.8
rpsL	30S ribosomal protein S12	<0.01	-1.8
MAB_0652	Hypothetical protein	<0.01	-1.8
MAB_2684c	Probable biotin synthase BioB	<0.01	-1.8
MAB_2716c	Probable manganese transport transmembrane protein	<0.01	-1.8
MAB_2327c	Hypothetical protein	0.03	-1.8
MAB_4647	Putative transcriptional regulator, TetR family	<0.01	-1.8
MAB_4903	Hypothetical protein	<0.01	-1.8
MAB_4401	Putative surface layer protein	0.02	-1.8
MAB_0208	Putative MarR-family transcriptional regulator	<0.01	-1.8
MAB_4646	Hypothetical protein	<0.01	-1.8
MAB_0653	Hypothetical MoxR-like ATPase	<0.01	-1.8
MAB_1055c	Conserved hypothetical protein (peptidase?)	<0.01	-1.7
MAB_2422c	Hypothetical protein	<0.01	-1.7
MAB_4557c	LemA family protein	<0.01	-1.7
MAB_3497	Hypothetical protein	<0.01	-1.7

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APPENDIX J

Significantly down-regulated genes (q-value <0.05) in 7C (continued)

Gene	Gene product	q-value	Fold change
MAB_2686c	Dethiobiotin synthetase (BioD)	0.05	-1.7
MAB_0321c	Hypothetical protein	<0.01	-1.7
MAB_3867	Hypothetical protein	0.02	-1.7
MAB_4700c	Hypothetical protein	<0.01	-1.7
MAB_4274c	Hypothetical protein	<0.01	-1.7
MAB_3996	Hypothetical protein	<0.01	-1.7
MAB_1130	Hypothetical protein	<0.01	-1.7
MAB_2067c	Probable acetyl/propionyl-CoA carboxylase beta subunit AccD2	<0.01	-1.7
MAB_1102	Hypothetical protein	<0.01	-1.7
MAB_3753c	Hypothetical protein	<0.01	-1.7
MAB_3946c	Putative short chain dehydrogenase/reductase	<0.01	-1.7
MAB_4003c	Putative UDP-glucose 4-epimerase GalE1	<0.01	-1.7
MAB_2687c	8-amino-7-oxononanoate synthase (BioF)	<0.01	-1.7
MAB_4067	Hypothetical protein	<0.01	-1.7
MAB_2496	Probable acyl-CoA dehydrogenase	0.05	-1.7
MAB_4479	Putative transcriptional regulator, TetR family	<0.01	-1.7
MAB_4697	Hypothetical protein	<0.01	-1.7
MAB_0518c	Inorganic pyrophosphatase	<0.01	-1.7
MAB_4828c	Hypothetical protein	<0.01	-1.7
MAB_3225	Putative lipoprotein LppW precursor	<0.01	-1.6
MAB_2152	Putative transcriptional regulator, TetR family	<0.01	-1.6

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APPENDIX J

Significantly down-regulated genes (q-value <0.05) in 7C (continued)

Gene	Gene product	q-value	Fold change
rplY	50S ribosomal protein L25/general stress protein Ctc	<0.01	-1.6
MAB_4833c	Hypothetical protein	0.02	-1.6
MAB_1086	Putative UTP-glucose-1-phosphate uridylyltransferase (GalU)	<0.01	-1.6
MAB_1012c	Putative YrbE family protein	<0.01	-1.6
MAB_4535c	Hypothetical protein	0.02	-1.6
MAB_0091	Putative ferredoxin-dependent glutamate synthase	<0.01	-1.6
rplM	50S ribosomal protein L13	0.01	-1.6
MAB_3754c	Hypothetical protein	<0.01	-1.6
MAB_1974	Putative secreted protein	<0.01	-1.6
MAB_4740	Possible beta-1,3-glucanase	<0.01	-1.6
MAB_0532	Hypothetical protein	<0.01	-1.6
MAB_3143c	Possible multifunctional enzyme siroheme synthase CysG/Uroporphyrin-III C-methyltransferase-like	<0.01	-1.6
MAB_1283c	Hypothetical protein	<0.01	-1.6
MAB_1390c	Hypothetical UbiE/COQ5 methyltransferase	<0.01	-1.6
MAB_0523	Hypoxanthine phosphoribosyltransferase (HPT)	<0.01	-1.6
MAB_2450	Probable undecaprenyl-diphosphatase	<0.01	-1.6
MAB_4824c	Hypothetical protein	<0.01	-1.6
MAB_2272c	Putative transcriptional regulator, GntR	<0.01	-1.6
MAB_4869	Hypothetical protein	<0.01	-1.6

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APPENDIX J

Significantly down-regulated genes (q-value <0.05) in 7C (continued)

Gene	Gene product	q-value	Fold change
MAB_1298c	Hypothetical protein	<0.01	-1.6
MAB_0022c	Probable permease	<0.01	-1.6
MAB_4905	Hypothetical protein	<0.01	-1.6
MAB_4403c	Probable acyl-[acyl-carrier protein] desaturase	<0.01	-1.6
MAB_1621	Probable nicotinate-nucleotide adenylyltransferase	0.04	-1.6
rpmA	50S ribosomal protein L27	<0.01	-1.6
MAB_2981c	Putative lipoprotein LppU	<0.01	-1.6
MAB_0007	Hypothetical protein	<0.01	-1.6
MAB_1542c	Probable amino acid permease	<0.01	-1.6
MAB_1402c	Putative lipoprotein LprE precursor	<0.01	-1.6
MAB_0347	Cell division control protein 48 CDC48	0.04	-1.6
MAB_3275	Probable cytochrome P450	0.04	-1.6
MAB_4062c	Hypothetical protein	<0.01	-1.6
MAB_4687	Putative transcriptional regulator, TetR family	0.01	-1.6
MAB_0859	Hypothetical protein	<0.01	-1.6
MAB_1652	Probable sulfate ABC transporter, sulfate-binding protein SubI	0.03	-1.6
MAB_3506c	Putative short-chain dehydrogenase/reductase	<0.01	-1.6
MAB_2792c	Probable aldehyde dehydrogenase	<0.01	-1.5
MAB_1596	Hypothetical protein	0.02	-1.5
MAB_1378c	Probable magnesium and cobalt transport transmembrane protein CorA	0.01	-1.5

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APPENDIX J

Significantly down-regulated genes (q-value <0.05) in 7C (continued)

Gene	Gene product	q-value	Fold change
rplN	50S ribosomal protein L14	<0.01	-1.5
MAB_2199c	Probable precorrin-8X methylmutase CobH	<0.01	-1.5
MAB_1459c	Conserved hypothetical protein (adenylate cyclase?)	<0.01	-1.5
MAB_2682c	Hypothetical protein	<0.01	-1.5
MAB_3050	Putative glutamate ABC transporter, ATP-binding protein	0.02	-1.5
MAB_0459c	Possible glycosyl hydrolase	0.01	-1.5
rnpA	Ribonuclease P protein component	<0.01	-1.5
MAB_2978	Hypothetical protein	<0.01	-1.5
MAB_4474	Hypothetical protein	<0.01	-1.5
MAB_3605	Hypothetical protein	<0.01	-1.5
MAB_4461	Hypothetical protein	<0.01	-1.5
MAB_2384	Hypothetical protein	<0.01	-1.5
MAB_0824	Putative L-carnitine dehydratase	<0.01	-1.5
rpsJ	30S ribosomal protein S10	<0.01	-1.5
MAB_3249	Hypothetical protein	<0.01	-1.5
MAB_0483c	Putative transcriptional regulator, TetR family	0.02	-1.5
MAB_0885c	Hypothetical lipoprotein lpqH precursor	<0.01	-1.5
MAB_1442	Probable peptide chain release factor 1 (RF-1)	<0.01	-1.5
MAB_0055c	Transcription regulator LysR family	<0.01	-1.5
MAB_3254c	Potassium-transporting ATPase A chain	<0.01	-1.5

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APPENDIX J

Significantly down-regulated genes (q-value <0.05) in 7C (continued)

Gene	Gene product	q-value	Fold change
MAB_2309c	Putative transcriptional regulator	<0.01	-1.5
MAB_1311	Hypothetical protein	0.04	-1.5
MAB_0242	Hypothetical protein	<0.01	-1.5
MAB_1031c	Probable manganese transport protein MntH	<0.01	-1.5
MAB_4805	Beta-lactamase-like hypothetical protein	<0.01	-1.5
MAB_0243	Hypothetical protein	<0.01	-1.5
MAB_2737c	Probable enoyl-CoA hydratase/isomerase	<0.01	-1.5
MAB_4133c	Hypothetical protein	<0.01	-1.5
MAB_4156c	3-oxoacyl-ACP reductase	<0.01	-1.5
rpsI	30S ribosomal protein S9	<0.01	-1.5
MAB_1145c	Putative lipoprotein LpqT precursor	<0.01	-1.5
MAB_3416	Probable membrane transport protein	0.02	-1.5
MAB_0339	Hypothetical protein	<0.01	-1.5
MAB_4077	Hypothetical protein	<0.01	-1.5
MAB_0577c	Putative ABC transporter solute binding protein	<0.01	-1.5
MAB_4780	(R)-hydratase	<0.01	-1.5
MAB_3201	Putative membrane protein MmpL	0.03	-1.5
MAB_0307c	Hypothetical protein	<0.01	-1.5
MAB_3008	Probable polyphosphate glucokinase PpgK	<0.01	-1.5
MAB_3674	Probable succinate dehydrogenase, hydrophobic membrane anchor protein SdhD	<0.01	-1.5

∞ indicates that the gene was expressed in *M. abscessus* ATCC 19977 but not in 7C.

APPENDIX J

Significantly down-regulated genes (q-value <0.05) in 7C (continued)

Gene	Gene product	q-value	Fold change
MAB_1201c	Hypothetical transcription elongation factor GreA	<0.01	-1.5
MAB_4938	Possible alternative RNA polymerase sigma factor SigM	<0.01	-1.5
MAB_3757	Hypothetical protein	<0.01	-1.5
MAB_3067c	Hypothetical protein	<0.01	-1.5
MAB_1144c	Probable short-chain dehydrogenase/reductase	<0.01	-1.5
MAB_2356	Hypothetical protein	<0.01	-1.5
MAB_3262c	Hypothetical protein	<0.01	-1.5
MAB_1101	Hypothetical protein	<0.01	-1.5
MAB_2881c	Hypothetical protein	<0.01	-1.5
MAB_0177	Antigen 85-A/B/C precursor	<0.01	-1.5
MAB_2689	Acyltransferase	<0.01	-1.5
MAB_4950c	Putative chromosome partitioning protein/ cobyrinic acid a,c-diamide synthase	<0.01	-1.5
MAB_0884c	Hypothetical protein	<0.01	-1.5
MAB_3369	Putative integral membrane amino acid transport protein	<0.01	-1.5
MAB_2051	Conserved hypothetical protein (thiolase?)	0.01	-1.5
MAB_4698	Hypothetical protein	0.01	-1.5
MAB_3517	Putative hydrolase, alpha/beta fold LipV	<0.01	-1.5
MAB_4476c	Putative monooxygenase	0.03	-1.5
MAB_3184	Hypothetical protein	<0.01	-1.5

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APPENDIX J

Significantly down-regulated genes (q-value <0.05) in 7C (continued)

Gene	Gene product	q-value	Fold change
MAB_4247	Putative dicarboxylate carrier protein	0.03	-1.5
MAB_0168c	Putative N-acetymuramoyl-L-alanine amidase	<0.01	-1.5
MAB_3934c	Possible hydrolase, alpha/beta fold	<0.01	-1.5
MAB_3759c	Hypothetical protein	<0.01	-1.5
MAB_4379c	MaoC-like dehydratase	0.04	-1.5
MAB_2114c	Luciferase-like	<0.01	-1.5
MAB_4093	Putative allophanate hydrolase	0.02	-1.5
MAB_0305	Putative aminotransferase	<0.01	-1.5
MAB_1840c	Putative beta-glucanase	<0.01	-1.5
MAB_0129c	Abortive infection protein	<0.01	-1.5
MAB_4823c	Hypothetical protein	0.04	-1.5
MAB_2182c	Hypothetical glycosyl transferase	<0.01	-1.5
MAB_4004c	Conserved hypothetical protein (excisionase?)	<0.01	-1.5
MAB_0158c	ABC transporter, ATP-binding protein	<0.01	-1.4
MAB_3364	Hypothetical protein	0.03	-1.4
MAB_2310	Possible drug efflux membrane protein	0.03	-1.4
MAB_4701	Hypothetical protein	<0.01	-1.4
MAB_0582c	Hypothetical protein	<0.01	-1.4
MAB_0861	Hypothetical protein	<0.01	-1.4
rpsR.1	30S ribosomal protein S18	<0.01	-1.4
MAB_4777c	Possible cellulase CelA (endoglucanase)	<0.01	-1.4
MAB_0175	Antigen 85-C precursor	<0.01	-1.4
MAB_3093c	Hypothetical protein	0.03	-1.4

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APPENDIX J

Significantly down-regulated genes (q-value <0.05) in 7C (continued)

Gene	Gene product	q-value	Fold change
rplC	50S ribosomal protein L3	<0.01	-1.4
MAB_4255	Hypothetical protein	<0.01	-1.4
MAB_3983c	Hypothetical protein	<0.01	-1.4
MAB_0025	Peptidyl-prolyl cis-trans isomerase	<0.01	-1.4
MAB_3223c	Probable signal peptidase I LepB	<0.01	-1.4
MAB_0426	Probable peptide ABC transporter DppA	0.02	-1.4
MAB_0108c	Hypothetical protein	<0.01	-1.4
MAB_0149c	Hypothetical protein	<0.01	-1.4
rplE	50S ribosomal protein L5	<0.01	-1.4
MAB_4560	Alcohol dehydrogenase B (AdhB)	0.01	-1.4
MAB_4885	Hypothetical protein	0.02	-1.4
MAB_0782	Hypothetical protein	<0.01	-1.4
MAB_4186c	Hypothetical protein	<0.01	-1.4
MAB_4452	Putative transcriptional regulator, MerR family	<0.01	-1.4
MAB_0301	Putative carboxylesterase	0.01	-1.4
MAB_4924	Hypothetical protein	<0.01	-1.4
MAB_2112	Hypothetical protein	<0.01	-1.4
dnaA	Chromosomal replication initiator protein DnaA	<0.01	-1.4
MAB_0478	Probable membrane protein, MmpL	<0.01	-1.4
MAB_0822	Probable acyl-CoA dehydrogenase	<0.01	-1.4
MAB_4088c	Possible mycolic acid synthase UmaA1	<0.01	-1.4
rplU	50S ribosomal protein L21	<0.01	-1.4
MAB_4827c	Hypothetical protein	<0.01	-1.4

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APPENDIX J

Significantly down-regulated genes (q-value <0.05) in 7C (continued)

Gene	Gene product	q-value	Fold change
MAB_3281	Putative enoyl-CoA hydratase/isomerase	<0.01	-1.4
MAB_4264c	Hypothetical protein	<0.01	-1.4
MAB_0411c	Putative anion transporter ATPase	0.02	-1.4
MAB_1533c	Probable oligoribonuclease	<0.01	-1.4
MAB_4309c	Putative serine protease	0.01	-1.4
MAB_1460	Hypothetical protein	<0.01	-1.4
MAB_2313	Hypothetical protein	0.02	-1.4
MAB_0877c	Hypothetical protein	<0.01	-1.4
rpsB	30S ribosomal protein S2	<0.01	-1.4
MAB_4703c	Probable membrane protein, MmpL	<0.01	-1.4
MAB_0610	Hypothetical protein	0.04	-1.4
MAB_1076	Mycobacterial persistence regulator MrpA (two component response transcriptional regulatory protein)	<0.01	-1.4
MAB_1367c	Hypothetical protein	<0.01	-1.4
MAB_4297c	Deoxycytidine triphosphate deaminase Dcd	<0.01	-1.4
MAB_4398c	Hypothetical protein	<0.01	-1.4
MAB_0732c	Hypothetical protein	<0.01	-1.4
MAB_1466c	Possible lipoprotein peptidase LpqM	<0.01	-1.4
MAB_3684c	Putative exodeoxyribonuclease	<0.01	-1.4
MAB_0757	Hypothetical protein	<0.01	-1.4
MAB_4451c	Conserved hypothetical protein (hydrolase?)	0.04	-1.4
MAB_4688	Hypothetical protein	0.03	-1.4
MAB_0781	Hypothetical protein	0.02	-1.4

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APPENDIX J

Significantly down-regulated genes (q-value <0.05) in 7C (continued)

Gene	Gene product	q-value	Fold change
MAB_2874	Probable peptidyl-prolyl cis-trans isomerase	<0.01	-1.4
MAB_3687	Probable o-acetylhomoserine sulfhydrylase MetC (homocysteine synthase)	0.03	-1.4
MAB_4743c	Putative dehydrogenase/reductase	0.04	-1.4
MAB_4194c	Hypothetical protein	0.04	-1.4
MAB_0416c	Putative Crp/Fnr-family transriptional regulator	<0.01	-1.4
MAB_3321c	Ketol-acid reductoisomerase	<0.01	-1.4
rpmE.1	50S ribosomal protein L31	<0.01	-1.4
MAB_3998	Hypothetical protein	<0.01	-1.4
rpsP	30S ribosomal protein S16	<0.01	-1.4
MAB_4702c	Hypothetical protein	<0.01	-1.4
MAB_4801	Possible twin-arginine translocation pathway	<0.01	-1.4
MAB_3480	Putative short chain dehydrogenase/reductase	0.03	-1.4
MAB_3784c	Preprotein translocase secY subunit	0.01	-1.4
MAB_0579c	Putative transcriptional regulator, TetR family	0.03	-1.4
MAB_2868c	Hypothetical protein	0.01	-1.4
MAB_2779c	Glyceraldehyde-3-phosphate dehydrogenase, type I	<0.01	-1.4
MAB_3404c	Probable ribonucleoside-diphosphate reductase beta subunit	<0.01	-1.4
MAB_1020	Probable undecaprenyl-phosphate galactosephosphotransferase	0.03	-1.4

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APPENDIX J

Significantly down-regulated genes (q-value <0.05) in 7C (continued)

Gene	Gene product	q-value	Fold change
MAB_2347c	Hypothetical protein	0.03	-1.4
MAB_2108	Probable undecaprenyl-diphosphatase (Bacitracin resistance protein)	<0.01	-1.4
MAB_1929	Hypothetical protein	<0.01	-1.4
MAB_1612	Probable GTP1/Obg-family GTP-binding protein	<0.01	-1.4
MAB_0383c	Putative transcriptional regulator, PadR-like	<0.01	-1.4
MAB_4729c	Putative Na+-dependent transporter	<0.01	-1.4
MAB_1979	Hypothetical protein	0.05	-1.4
MAB_1266	Probable GTP binding protein	<0.01	-1.4
MAB_4867c	Probable dCTP deaminase/DeoxyUTP pyrophosphatase	<0.01	-1.4
MAB_1551c	Hypothetical protein	0.03	-1.4
MAB_0983c	Probable alcohol dehydrogenase, zinc-containing	<0.01	-1.4
MAB_0850	Probable acyl-coa thiolase FadA	<0.01	-1.4
MAB_4278	Hypothetical protein	<0.01	-1.4
MAB_4250	Hypothetical protein	0.03	-1.4
MAB_0470c	Putative hydrolase (alpha/beta hydrolase fold)	0.03	-1.4
MAB_2993c	Hypothetical protein	<0.01	-1.4
MAB_4711	Hypothetical protein	0.03	-1.4
MAB_0743c	Hypothetical protein	0.02	-1.4
MAB_3897c	Hypothetical protein	0.04	-1.4
gidB	16S rRNA methyltransferase G	<0.01	-1.4

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APPENDIX J

Significantly down-regulated genes (q-value <0.05) in 7C (continued)

Gene	Gene product	q-value	Fold change
MAB_2958	Putative transmembrane-transport protein	<0.01	-1.4
MAB_3424c	Hypothetical protein	0.04	-1.3
MAB_4253	Hypothetical protein	<0.01	-1.3
rplJ	50S ribosomal protein L10	<0.01	-1.3
MAB_1061	34 kDa antigenic protein homolog	<0.01	-1.3
MAB_0192c	Probable oxidoreductase	<0.01	-1.3
MAB_4826	Conserved hypothetical protein (helicase?)	<0.01	-1.3
MAB_2375	Hypothetical protein	<0.01	-1.3
MAB_2391	Hypothetical protein	0.03	-1.3
MAB_4096c	Hypothetical protein	<0.01	-1.3
MAB_4562	Putative lipoprotein LprO	<0.01	-1.3
MAB_1139	4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol kinase	<0.01	-1.3
MAB_3185	Hypothetical protein	0.04	-1.3
rpsF	30S ribosomal protein S6	<0.01	-1.3
MAB_4946	Putative transcriptional regulator, TetR	0.04	-1.3
MAB_1142c	Probable peptidyl-tRNA hydrolase (PTH)	0.01	-1.3
MAB_3188c	Uridylate kinase PyrH	<0.01	-1.3
MAB_1456c	Conserved hypothetical protein (cobalamin adenosyltransferase?)	<0.01	-1.3
MAB_4704c	Probable membrane protein, MmpL	<0.01	-1.3
MAB_3760	Hypothetical protein	<0.01	-1.3
MAB_4774c	Hypothetical protein	<0.01	-1.3

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APPENDIX J

Significantly down-regulated genes (q-value <0.05) in 7C (continued)

Gene	Gene product	q-value	Fold change
MAB_0511c	Integral membrane protein TerC (tellurium resistance)	0.05	-1.3
MAB_1549c	Putative RNA binding protein, contains S1 domain	<0.01	-1.3
MAB_0405c	Hypothetical protein	<0.01	-1.3
MAB_4251	Conserved hypothetical protein (peptidase?)	<0.01	-1.3
MAB_2295c	Hypothetical protein	0.01	-1.3
MAB_0724c	Putative amidase AmiC	0.02	-1.3
MAB_3777	Band 7 protein	<0.01	-1.3
MAB_3681	Probable penicillin-binding protein DacB1	0.02	-1.3
MAB_1164	Putative conserved lipoprotein LpqU	0.01	-1.3
MAB_3098	Probable transmembrane carbonic anhydrase	<0.01	-1.3
MAB_4898c	Single-stranded DNA-binding protein	<0.01	-1.3
MAB_4187	Peptide deformylase	<0.01	-1.3
MAB_0966	Hypothetical protein	0.04	-1.3
MAB_1200	Hypothetical protein	0.02	-1.3
MAB_4817	Hypothetical protein	<0.01	-1.3
MAB_2627c	Possible two-component response regulatory protein	<0.01	-1.3
MAB_3991c	Possible Uroporphyrin-III C-methyltransferase	0.01	-1.3
MAB_0531c	Hypothetical protein	0.01	-1.3
MAB_1580	Trigger factor (TF)	<0.01	-1.3

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APPENDIX J

Significantly down-regulated genes (q-value <0.05) in 7C (continued)

Gene	Gene product	q-value	Fold change
MAB_0710	Hypothetical protein	<0.01	-1.3
MAB_0267	Hypothetical protein	0.02	-1.3
MAB_4376c	Putative acetoacetyl-CoA synthetase	0.01	-1.3
MAB_0164	Probable short chain dehydrogenase/reductase	<0.01	-1.3
MAB_0376	Conserved hypothetical protein (peptidase?)	<0.01	-1.3
MAB_4888	Hypothetical protein	0.03	-1.3
MAB_4693	Probable cytochrome P450	0.03	-1.3
MAB_3912	Hypothetical protein	<0.01	-1.3
MAB_4741c	Putative terminal quinol oxidase, subunit I	0.03	-1.3
MAB_1300c	Hypothetical protein	0.01	-1.3
MAB_0409	Putative transcriptional regulator WhiB	<0.01	-1.3
MAB_1553c	Hypothetical protein	0.02	-1.3
MAB_1302	Hypothetical protein	0.01	-1.3
MAB_0482c	Hypothetical protein	0.03	-1.3
MAB_1673	Hypothetical protein	0.05	-1.3
MAB_4111c	Putative epimerase/dehydratase	<0.01	-1.3
MAB_2324	Possible tRNA/rRNA methyltransferase	<0.01	-1.3
MAB_1409c	Putative drug antiporter protein precursor	0.03	-1.3
MAB_3252c	Potassium-transporting ATPase C chain	0.02	-1.3
MAB_3896c	Hypothetical protein	<0.01	-1.3

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APPENDIX J

Significantly down-regulated genes (q-value <0.05) in 7C (continued)

Gene	Gene product	q-value	Fold change
MAB_2101	Probable recombinase	<0.01	-1.3
MAB_1004c	Hypothetical protein	<0.01	-1.3
MAB_1147c	Probable ribose-phosphate pyrophosphokinase RppK (PRPP synthetase)	<0.01	-1.3
MAB_2973c	Putative methyltransferase	0.01	-1.3
MAB_2065c	Probable acyl-CoA dehydrogenase FadE	<0.01	-1.3
MAB_2325	Putative short-chain dehydrogenase/reductase	<0.01	-1.3
rplD	50S ribosomal protein L4	0.03	-1.3
MAB_0342c	Putative nitroreductase family protein	0.03	-1.3
MAB_0752c	Putative transcriptional regulator	<0.01	-1.3
MAB_4508	Putative membrane protein, MmpL	0.02	-1.3
MAB_3009	Probable RNA polymerase sigma factor RpoD (Sigma-A)	0.01	-1.3
MAB_4058c	Hypothetical protein	0.04	-1.3
MAB_3292c	DNA-binding protein HU homolog (Histone-like)	0.03	-1.3
MAB_2888c	Hypothetical protein	0.02	-1.3
MAB_3823	Pyridoxamine 5'-phosphate oxidase	0.01	-1.3
MAB_2398	Putative phosphatidylglycerophosphate synthase PgsA2	0.02	-1.3
MAB_0728	Probable phosphoribosylformylglycinamidine cyclo-ligase PurM	<0.01	-1.3

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APPENDIX J

Significantly down-regulated genes (q-value <0.05) in 7C (continued)

Gene	Gene product	q-value	Fold change
MAB_2824c	Putative integration host factor (MihF)	<0.01	-1.3
MAB_3774c	Translation initiation factor IF-1	0.02	-1.3
MAB_2977	Hypothetical protein	<0.01	-1.3
MAB_4734	Probable dehydrogenase	0.03	-1.3
MAB_2377	Hypothetical protein	0.02	-1.3
MAB_3765	Probable cutinase cut3 precursor	0.02	-1.3
MAB_1125c	Hypothetical acetyltransferase, GNAT family	0.05	-1.3
MAB_3663	Hypothetical protein	0.02	-1.3
MAB_3871c	Possible ribonucleotide ABC transporter, ATP-binding	0.02	-1.3
MAB_2161c	Hypothetical low molecular weight antigen Mtb12	0.03	-1.3
MAB_1328	Probable aminotransferase	0.01	-1.3
MAB_3790	Hypothetical protein	0.04	-1.3
MAB_3614	Hypothetical protein	0.04	-1.3
rplI	50S ribosomal protein L9	0.02	-1.3
MAB_2402	Glycine cleavage system H protein (GcvH)	0.02	-1.3
MAB_1975	Hypothetical protein	0.01	-1.3
MAB_2638c	acyl-CoA thioesterase II	0.04	-1.3
MAB_1607	Possible ribonuclease E Rne	0.03	-1.3
MAB_3040c	Probable acyl-CoA dehydrogenase	0.02	-1.3
MAB_3363c	Electron transfer flavoprotein beta-subunit FixA	0.02	-1.3
MAB_4008c	Hypothetical protein	0.03	-1.3

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APPENDIX J

Significantly down-regulated genes (q-value <0.05) in 7C (continued)

Gene	Gene product	q-value	Fold change
MAB_3294c	3-isopropylmalate dehydratase large subunit	0.02	-1.3
MAB_1003c	Hypothetical protein	0.02	-1.3
MAB_4926	Hypothetical protein	0.02	-1.3
MAB_3475c	Putative cell division ATP-binding protein FtsE	0.02	-1.3
MAB_3718c	GMP synthase [glutamine-hydrolyzing]	0.02	-1.3
MAB_0401	Acyltransferase	0.03	-1.3
MAB_1351c	Putative glycosyltransferase	0.03	-1.3
MAB_0024c	Hypothetical protein	0.04	-1.3
MAB_1025	Hypothetical protein	0.03	-1.3
MAB_4254c	Fructose-bisphosphate aldolase	0.02	-1.3
MAB_4005c	Probable pyrroline-5-carboxylate reductase ProC	0.03	-1.3
MAB_1082c	Hypothetical large-conductance mechanosensitive channel	0.02	-1.3
MAB_4524	Hypothetical protein	0.02	-1.3
MAB_1869c	Probable zinc-dependent alcohol dehydrogenase AdhE2	0.03	-1.3
MAB_1933c	Probable glutamine synthetase, type I (GlnA1)	0.03	-1.3
MAB_1389c	Probable short-chain dehydrogenase/reductase	0.04	-1.3
rplL	50S ribosomal protein L7/L12	0.03	-1.3
MAB_1052c	Hypothetical protein	0.03	-1.3
MAB_3105c	Hypothetical protease	0.03	-1.3

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APPENDIX J

Significantly down-regulated genes (q-value <0.05) in 7C (continued)

Gene	Gene product	q-value	Fold change
MAB_3167c	Penicillin-binding protein, putative	0.03	-1.3
MAB_2321	Translation initiation factor IF-3 (InfC)	0.03	-1.3
MAB_2413c	6-phosphogluconate dehydrogenase	0.03	-1.3
MAB_2975c	AFG1-like ATPase	0.04	-1.3
MAB_3930c	Ubiquinone/menaquinone biosynthesis methlytransferase UbiE	0.04	-1.3
MAB_2378c	Hypothetical protein	0.04	-1.3
MAB_0783c	Bacteriophage protein	0.04	-1.2
MAB_1189c	Probable methylmalonic acid semialdehyde dehydrogenase MmsA	0.03	-1.2
MAB_0039c	Hypothetical protein	0.05	-1.2
rpsH	30S ribosomal protein S8	0.04	-1.2
MAB_3454	Hypothetical protein	0.04	-1.2
MAB_1280c	Hypothetical protein	0.05	-1.2
MAB_3503	Putative ABC transporter, ATP-binding protein	0.04	-1.2
MAB_3895c	Probable preprotein translocase SecE subunit	0.04	-1.2
MAB_1919	Conserved hypothetical protein (possible hydrolase)	0.04	-1.2
MAB_2645c	Indole-3-glycerol phosphate synthase TrpC	0.04	-1.2
rplT	50S ribosomal protein L20	0.04	-1.2
MAB_1447	ATP synthase A chain AtpB	0.04	-1.2

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

The findings from this study generated two manuscripts. The first manuscript has been published in the Journal of Medical Microbiology. The second manuscript has been submitted to International Journal of Medical Microbiology. In addition, a part of this study's data was presented at the Inaugural FMHS Scientific Meeting (UTAR Sungai Long, Malaysia), 2017. The references of these papers or publications are as follows:

Ng, H.F. et al., 2018. A mutation in anti-sigma factor MAB_3542c may be responsible for tigecycline resistance in *Mycobacterium abscessus*. *Journal of Medical Microbiology*, 67(12), pp.1676–1681.

Ng, H.F. et al., 2017. Characterization of a tigecycline-selected mutant of *Mycobacterium abscessus*. In *Inaugural FMHS Scientific Meeting*. Sungai Long: Universiti Tunku Abdul Rahman, p. 46.