

### **Supplementary figure legends**

**Supplementary Fig. 1.** Gross of appearance of rabbits from mock-infected group (i.e., inoculated with plain culture medium). No pathological sign was observed in rabbits inoculated with plain culture medium up to day 3 PI.

**Supplementary Fig. 2.** Growth of the *tmyt* knockout mutants are unimpaired *in vitro*. The *T. pulmonis* PW1004-WT and the 3 *tmyt* knockout mutants were grown as described in Materials and Methods, and OD<sub>600</sub> measurements were made on 1-ml aliquots taken at indicated time intervals. There was no statistical difference among them.

**Supplementary Fig. 3.** Appearance of the liquid cultures of the PW1004-WT, *tmytC* knockout and complemented mutants after 48 h incubation at 37°C in an aerobic environment. A ring of cells (boxed in yellow) adhering at the air-liquid interface was observed in the culture tubes of the PW1004-WT and PW1004Δ*tmytC*/*ptmytC*, but not those of PW1004Δ*tmytC*. In contrast, a large cell pellet (boxed in green) was observed in the culture tube of PW1004Δ*tmytC*, suggesting its weaker adhesion to plastics.

**Supplementary Table 1. Primers used in this study**

Primers	Sequence (5' to 3') <sup>a</sup>	Restriction site
Primers for mutagenesis of <i>tmytA</i>		
<i>tmytA</i> -UF-1	GTGCCCCGGCGCCGCTGA	
<i>tmytA</i> -DR-1	TGATCTGCGCGACCGACCGGT	
<i>tmytA</i> -UF	<u>CCCAAGCTT</u> GTGCCCCGGCGCCGCTGA	<i>Hind</i> III
<i>tmytA</i> -UR	<u>CCCAAGCTT</u> CATGGGAACAGTGTTACACGGGTGT	<i>Hind</i> III
<i>tmytA</i> -DF	<u>CGCGGATCC</u> GACGACCGGCCGGGGTT	<i>Bam</i> HI
<i>tmytA</i> -DR	<u>CGCGGATCC</u> TGATCTGCGCGACCGACCGGT	<i>Bam</i> HI
Inner- <i>tmytA</i> -F	GCGAGCGCGAGCCCGTG	
Inner- <i>tmytA</i> -R	GTGGCCGGCAGCGACGT	
Primers for mutagenesis of <i>tmytB</i>		
<i>tmytB</i> -UF-1	GCGGCGATGGTGCGAAGAGA	
<i>tmytB</i> -DR-1	TGTGGGTGCCACCGACGAGAAGT	
<i>tmytB</i> -UF	<u>CCCAAGCTT</u> TGGCGCGATGGTGCGAAGAGA	<i>Hind</i> III
<i>tmytB</i> -UR	<u>CCCAAGCTT</u> TCTTGGCCAGCCGTTGCGCAT	<i>Hind</i> III
<i>tmytB</i> -DF	<u>CGCGGATCC</u> TGGGGCTACTGGCAGGATCA	<i>Bam</i> HI
<i>tmytB</i> -DR	<u>CGCGGATCC</u> GGTGCCACCGACGAGAAGT	<i>Bam</i> HI
Inner- <i>tmytB</i> -F	ATTCGGACGGAAAAGCACCTGCTGTA	
Inner- <i>tmytB</i> -R	AGCCTTGTCACAGCCGTCACTCT	
Primers for mutagenesis of <i>tmytC</i>		
<i>tmytC</i> -UF-1	GTCTGCGCGCCACCTGGGA	
<i>tmytC</i> -DR-1	GCAGGCCCTTGGATCCGACGGT	
<i>tmytC</i> -UF	<u>CCCAAGCTT</u> GTCTCGCTGGTCGGCCTGTCTG	<i>Hind</i> III
<i>tmytC</i> -UR	GGTCGCTCCCCAGGCTGCTCGATTGCT	
<i>tmytC</i> -DF	GCAGCCTGGGGGAGCGACCAGGTCTGGCAGATG	
<i>tmytC</i> -DR	<u>CCCAAGCTT</u> CAGGCCCTTGGATCCGACGGT	<i>Hind</i> III
Inner- <i>tmytC</i> -F	AGGAAACCGGGAGTGTCTGTAACGC	
Inner- <i>tmytC</i> -R	AGGGGCTCGTCGACGTCGGT	
Primers for expression of <i>tmytC</i>		
<i>hsp60</i> -F	<u>CCAAGCTT</u> ACGCTCGACTCCAATACGGGGGT	<i>Hind</i> III
<i>hsp60</i> -R	GGCTGCTCGCATTCGATACCTCCTTTTGCGAAGTGATTCCCT	
<i>tmytC</i> -F	GGAGGTATCGAATGCGAGCAGCCTGGGGTAAGC	
<i>tmytC</i> -R	<u>CTAGTCTAGAT</u> CAGCTGTTGAACCAGCCGGTG	<i>Xba</i> I

<sup>a</sup>Restriction sites in the primer sequences are in underlined.

**Supplementary Table 2. Putative genes related to biofilm formation in *T. tyrosinosolvens*-PW899 and *T. pulmonis*-PW1004 respectively**

GenBank accession number of PW899 (PW1004)	Gene	Protein	Bacteria with the closest matching sequences to PW899 (PW1004)	Amino acid identities to PW899 (PW1004)	Pathways involved	References
RDB46325.1 (RDH10422.1)	<i>tmytA</i>	Mycolyltransferase	<i>W. limnetica</i> ( <i>W. limnetica</i> )	45% (45%)	Cell wall synthesis	Ojha et al.[1]
RDB46324.1 (RDH10421.1)	<i>tmytB</i>	Mycolyltransferase	<i>W. limnetica</i> ( <i>W. limnetica</i> )	44% (44%)	Cell wall synthesis	Ojha et al.[1]
RDB46325.1 (RDH10422.1)	<i>tmytC</i>	Mycolyltransferase	<i>W. limnetica</i> ( <i>W. limnetica</i> )	45% (45%)	Cell wall synthesis	Ojha et al.[1]
RDB46803.1 (RDH10071.1)	<i>smeg1529</i>	Serine esterase	<i>Mycolicibacterium aromaticivorans</i> ( <i>M. aromaticivorans</i> )	44% (42%)	Cell wall synthesis	Ojha et al.[1]
RDB46314.1 (RDH10413.1)	<i>pks16</i>	Putative polyketide synthase	<i>Gordonia kroppenstedtii</i> ( <i>G. kroppenstedtii</i> )	73% (72%)	Lipid synthesis	Ojha et al.[2]
RDB47756.1 (RDH09883.1)	<i>fadD26</i>	Fatty-acid-CoA ligase	<i>Gordonia aichiensis</i> ( <i>G. aichiensis</i> )	75% (73%)	Lipid synthesis	Mohandas et al.[3]
RDB45407.1 (RDH12857.1)	<i>papA5</i>	Phthiocerol/phthiodiolone dimycocerosyl transferase	<i>Gordonia effuse</i> ( <i>G. polyisoprenivorans</i> )	69% (62%)	Lipid synthesis	Mohandas et al.[3]
RDB49989.1 (RDH13721.1)	<i>mmpL11</i>	Mycobacterial membrane protein large	<i>Gordonia desulfuricans</i> ( <i>G. desulfuricans</i> )	73% (73%)	Lipid synthesis	Wright et al.[4]
RDB47405.1 (RDH10177.1)	<i>groEL1</i>	Heat shock protein 1	<i>Rhodococcus rhodochrous</i> ( <i>R. rhodochrous</i> )	75% (75%)	Mycolic acid synthesis	Ojha et al.[5]
RDB48772.1 (RDH09310.1)	<i>mmaa4</i>	Methoxy mycolic acid synthase	<i>Mycobacterium avium</i> complex ( <i>Rhodococcus zopfii</i> )	64% (70%)	Mycolic acid synthesis	Sambandan et al.[6]
RDB45065.1 (RDH10897.1)	<i>lsr2</i>	Leprosy serum reactive clone 2	<i>Mycobacteroides abscessus</i> ( <i>M. abscessus</i> )	64% (67%)	Mycolic acid synthesis	Chen et al.[7]

## References

1. Ojha AK, Trivelli X, Guerardel Y, et al. Enzymatic hydrolysis of trehalose dimycolate releases free mycolic acids during mycobacterial growth in biofilms. *The Journal of biological chemistry*. 2010 Jun 4;285(23):17380-9.
2. Ojha AK, Baughn AD, Sambandan D, et al. Growth of *Mycobacterium tuberculosis* biofilms containing free mycolic acids and harbouring drug-tolerant bacteria. *Molecular microbiology*. 2008 Jul;69(1):164-74.
3. Mohandas P, Budell WC, Mueller E, et al. Pleiotropic consequences of gene knockouts in the phthiocerol dimycocerosate and phenolic glycolipid biosynthetic gene cluster of the opportunistic human pathogen *Mycobacterium marinum*. *FEMS Microbiol Lett*. 2016 Mar;363(5):fnw016.
4. Wright CC, Hsu FF, Arnett E, et al. The *Mycobacterium tuberculosis* MmpL11 Cell Wall Lipid Transporter Is Important for Biofilm Formation, Intracellular Growth, and Nonreplicating Persistence. *Infection and immunity*. 2017 Aug;85(8).
5. Ojha A, Anand M, Bhatt A, et al. GroEL1: a dedicated chaperone involved in mycolic acid biosynthesis during biofilm formation in mycobacteria. *Cell*. 2005 Dec 2;123(5):861-73.
6. Sambandan D, Dao DN, Weinrick BC, et al. Keto-mycolic acid-dependent pellicle formation confers tolerance to drug-sensitive *Mycobacterium tuberculosis*. *mBio*. 2013 May 7;4(3):e00222-13.
7. Chen JM, German GJ, Alexander DC, et al. Roles of Lsr2 in colony morphology and biofilm formation of *Mycobacterium smegmatis*. *Journal of bacteriology*. 2006 Jan;188(2):633-41.