







Mycolyltransferase is important for biofilm formation and pathogenesis of *Tsukamurella* keratitis

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ABSTRACT

Tsukamurella, a group of multi-drug resistant, Gram-positive, aerobic, and partially acid-fast bacteria, are emerging causes of bacterial conjunctivitis and keratitis. However, the pathogenesis of *Tsukamurella* keratitis is largely unknown. To address this, we used New Zealand White rabbits to develop the first eye infection model and conducted *in vitro* tests to study the pathogenesis mechanisms of *Tsukamurella*. There is increasing evidence that biofilms play a significant role in ocular infections, leading us to hypothesize that biofilm formation is crucial for effective *Tsukamurella* infection. In order to look for potential candidate genes which are important in biofilm formation and *Tsukamurella* keratitis. We performed genome sequencing of two ocular isolates, *T. pulmonis*-PW1004 and *T. tyrosinosolvens*-PW899, to identify potential virulence factors. Through *in vitro* and *in vivo* studies, we characterized their biological roles in mediating *Tsukamurella* keratitis. Our findings confirmed that *Tsukamurella* is an ocular pathogen by fulfilling Koch's postulates, and using genome sequence data, we identified *tmytC*, encoding a mycolyltransferase, as a crucial gene in biofilm formation and causing *Tsukamurella* keratitis in the rabbit model. This is the first report demonstrating the novel role of mycolyltransferase in causing ocular infections. Overall, our findings contribute to a better understanding of *Tsukamurella* pathogenesis and provide a potential target for treatment. Specific inhibitors targeting TmytC could serve as an effective treatment option for *Tsukamurella* infections.

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Introduction

Using the information obtained from the analysis of 16S rRNA gene sequences, *Tsukamurella* was first proposed as a genus in 1988 [1], although the first strain of this group of bacteria was described in 1941 [2], and the first human isolate was reported in 1971 [3]. Similar to related genera of the order *Corynebacteriales*, such as *Nocardia*, *Rhodococcus*, and *Gordonia*, members of *Tsukamurella* are Gram-positive, aerobic,

catalase-positive, and partially acid-fast as a result of the presence of mycolic acid in the cell envelope. Due to their similar phenotypic properties, differentiation of these genera and speciation within these genera is difficult in most clinical microbiology laboratories [4]. Although over 21 "*Tsukamurella* species" have been described, using *in silico* genome-to-genome comparison as the standard of classification, only 16 *Tsukamurella* species should be included in

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this genus according to the current state of the taxonomy at the time of writing (<https://lpsn.dsmz.de/genus/tsukamurella>). Among these 16 species, 12 are known to be associated with human infections. Traditionally, the most commonly reported *Tsukamurella* infections in humans are indwelling device-related infections, such as catheter-related bacteremia and peritonitis associated with continuous ambulatory peritoneal dialysis [5–8].

In 2003 and 2009, we first reported *Tsukamurella* species as novel causes of bacterial conjunctivitis and keratitis respectively [9,10]. Since then, cases of *Tsukamurella* ocular infections have been noted in other countries [7,11–14]. Similar to keratitis caused by other bacteria, *Tsukamurella* keratitis is also associated with wearing contact lenses [9]. In the last few years, we have also discovered five additional novel *Tsukamurella* species, *T. sinensis*, *T. hongkongensis*, *T. ocularis*, *T. hominis* and *T. conjunctivitis*, from patients with ocular infections [15–17]. Moreover, our recent study, based on the largest number of *Tsukamurella* cases, showed that the number of *Tsukamurella* isolates recovered from ophthalmological specimens was higher than that recovered from blood cultures [18]. Although ocular infections could be the most important group of disease caused by *Tsukamurella*, the pathogenesis of *Tsukamurella* keratitis is largely unknown. We hypothesized that biofilm formation played a key role in *Tsukamurella* keratitis as well as other indwelling device infections. To test the hypothesis, in the first part of this study, we developed a rabbit model of *Tsukamurella* keratitis and sequenced and annotated the genomes of *T. tyrosinosolvens* and

T. pulmonis, which have been reported to cause keratitis, in order to identify candidate genes for biofilm formation. In the second part of the study, the candidate genes were studied through gene deletion and complementation experiments as well as *in vitro* and animal models developed.

Materials and methods

Ethics statement

This study was approved by the institutional review board of The University of Hong Kong (HKU)/Hospital Authority Hong Kong West Cluster in Hong Kong (IRB UW 16-365). The animal experiments were approved by the Committee on the Use of Live Animals in Teaching and Research (CULATR), HKU (CULATR 4794-18 and 4448-17) and the Department of Health, the Government of the HKSAR under the Animals (Control of Experiments) Ordinance, Chapter 340.

Bacterial strains and growth conditions

The source of all *Tsukamurella* strains used in this study is detailed in Table 1. *T. pulmonis*-PW1004 was isolated from the serous discharge of a 69-year-old Chinese woman with conjunctivitis, while *T. tyrosinosolvens*-PW899 was previously isolated from the corneal scraping of an 87-year-old Chinese woman with keratitis (Table 1) [9,10]. Unless otherwise specified, all *Tsukamurella* isolates were grown on brain heart infusion (BHI) agar at 37°C under aerobic conditions for 48 h (h).

Table 1. Bacterial strains and plasmids used in this study.

Strains or plasmids	Genotype and descriptions	Source or reference
<i>Strains</i>		
<i>E. coli</i> DH5a	F- ϕ 80 <i>lacZ</i> Δ <i>M15</i> Δ(<i>lacZYA-argF</i>) U169 <i>recA1 endA1 hsdR17</i> (rk-, mk+) <i>gal- phoA supE44 λ- thi-1 gyrA96 relA1</i>	Invitrogen
<i>T. tyrosinosolvens</i> PW899	Corneal scraping of a patient with keratitis	Woo et al. [9]
<i>T. pulmonis</i> PW1004	Eye swab of a patient with conjunctivitis	Woo et al. [10]
PW1004	PW1004 derivative with <i>galk</i> deletion, Δ <i>galk</i>	Present study
PW1004Δ <i>tmytA</i>	PW1004 derivative with <i>tmytA</i> deletion, Δ <i>galk</i>	Present study
PW1004Δ <i>tmytB</i>	PW1004 derivative with <i>tmytB</i> deletion, Δ <i>galk</i>	Present study
PW1004Δ <i>tmytC</i>	PW1004 derivative with <i>tmytC</i> deletion, Δ <i>galk</i>	Present study
<i>Plasmids</i>		
pCR®-XL-TOPO®	Cloning vector, pUC <i>ori kan</i>	Invitrogen
p2NIL	Suicidal plasmid, <i>oriE kan</i>	Parish et al. [46]
pNV18	<i>Tsukamurella/E. coli</i> shuttle vector, <i>aph</i>	Chiba et al. [30]
p2NIL-Galk	p2NIL expressing wild-type Galk under the control of the <i>hsp60</i> promoter, <i>kan</i>	Present study
pCR-XL- <i>tmytA</i>	pCR-XL containing <i>tmytA</i> and flanking fragments, <i>kan</i>	Present study
pCR-XL- <i>tmytB</i>	pCR-XL containing <i>tmytB</i> and flanking fragments, <i>kan</i>	Present study
pCR-XL- <i>tmytC</i>	pCR-XL containing <i>tmytC</i> and flanking fragments, <i>kan</i>	Present study
pΔ <i>tmytA</i>	p2NIL containing flanking fragments of <i>tmytA</i> , expressing Galk under the control of the <i>hsp60</i> promoter, <i>kan</i>	Present study
pΔ <i>tmytB</i>	p2NIL containing flanking fragments of <i>tmytB</i> , expressing Galk under the control of the <i>hsp60</i> promoter, <i>kan</i>	Present study
pΔ <i>tmytC</i>	p2NIL containing flanking fragments of <i>tmytC</i> , expressing Galk under the control of the <i>hsp60</i> promoter, <i>kan</i>	Present study
ptmytC	pNV18 expressing ORF of <i>tmytC</i> under the control of the <i>hsp60</i> promoter, <i>aph</i> , for TmytC protein expression	Present study

The bacterial cell density was determined by measuring the optical density at 600 nm (OD₆₀₀). The growth kinetics of the wild-type PW1004 (PW1004-WT), *tmytC* (*Tsukamurella mycolyltransferase C*) knockout and complemented mutants were examined by monitoring OD₆₀₀ at different time points.

Rabbit model for *Tsukamurella keratitis*

Eight male New Zealand White (NZW) rabbits, 1.5–2.0 kg, were inoculated intrastromally as described previously [19]. Corneas of four rabbits were injected with approximately 10 µl (i.e. 10⁶ CFU) of *T. pulmonis*-PW1004 using a microliter syringe with a 30G needle. Another four rabbit corneas were challenged with plain culture medium as the control. Rabbits were monitored daily for signs of disease. At 24 h post-infection (PI), rabbit eyes were photographed, evaluated for pathology, and sacrificed. After euthanasia, the corneas of each rabbit were surgically removed for histopathological and immunohistochemical analyses and bacterial enumeration.

Quantification of *Tsukamurella* cells

To quantify the number of *Tsukamurella* in the cornea, each tissue was homogenized and diluted in PBS. The diluted sample was plated in triplicate on BHI agar and incubated for 48 h at 37°C. The number of bacteria was expressed as CFU/cornea.

Histopathological and immunohistochemical analyses

Mice antiserum against *T. pulmonis*-PW1004 was produced by subcutaneously injecting 200 µl of heat-inactivated *Tsukamurella* cells (i.e. 10⁷ CFU) into three mice, using an equal volume of complete Freund's adjuvant (Sigma, USA) as described previously [20]. Two weeks after the last immunization, 100 µl of blood was collected via the lateral saphenous vein of the mice to obtain the sera. Serum samples collected from a mock-infected mouse were used as the control antibody.

To examine the histopathology of corneal tissues of rabbits challenged with the PW1004-WT and its mutants, excised infected eyes were examined. Corneal tissues were fixed, embedded, and stained with haematoxylin and eosin (H&E) according to Fischer et al. [21]. Histopathological changes were observed using an Olympus BX53 Digital Upright Microscope with DP80 microscope camera (Tokyo, Japan) and imaging system. Immunohistochemical staining for *Tsukamurella* was performed using anti-*T. pulmonis*-PW1004 serum using protocols as described previously [20]. Sections were counterstained with

haematoxylin. Tissues from sterile saline controls were included as negative controls.

Myeloperoxidase (MPO) assay

MPO is an enzyme used to measure polymorphonuclear leukocyte (PMN) accumulation in tissue samples. The amount of MPO activity in corneal homogenates can indicate the number of infiltrating PMNs. The assay uses a colorimetric reagent, o-dianisidine, to react with the hypochlorite produced by the MPO reaction. One MPO unit of activity is equivalent to approximately 100,000 PMNs [22].

Draft genome sequencing and analysis

The draft genomes of *T. tyrosinosolvens*-PW899 and *T. pulmonis*-PW1004 were sequenced using high-throughput sequencing and assembled as previously described [23]. Genomic DNA was extracted from overnight cultures of each *Tsukamurella* strain grown on BHI agar using the QIAGEN Genomic-tip 20/G kit, following the manufacturer's instructions (QIAGEN). The extracted DNA samples were then subjected to sequencing using the Illumina Hi-Seq 1500 platform, generating 151-bp paired-end reads. The sequence raw reads were processed using Trimmomatic v0.32 to remove adapters, low-quality reads and duplicate reads [24]. *De novo* assembly of the DNA sequences was performed using Velvet 1.2.10, with default parameters [25]. The resulting sequences were used for the prediction and annotation of protein coding regions. This was performed using version 2.0 of the RAST (Rapid Annotations using Subsystem Technology) server, applying default parameters and the RASTtk annotation scheme, and the Clusters of Orthologous Groups of proteins (COGs) [26,27]. Circular maps were generated using CGview [28]. This Whole Genome Shotgun project has been deposited in DDBJ/EMBL/GenBank under the accession numbers QPKD00000000; BioSample SAMN09691326 for *T. tyrosinosolvens*-PW899 and QQQF00000000; BioSample SAMN09691327 for *T. pulmonis*-PW1004.

Construction of non-polar deletion mutant strains

Bacterial strains and plasmids used in this part of the study are listed in Table 1. The wild-type *T. pulmonis*-PW1004 was a clinical strain isolated from a patient in Hong Kong [10]. Unmarked, non-polar deletion of *tmytA*, *tmytB*, and *tmytC* were constructed respectively by homologous recombination using our newly developed suicide plasmid p2NIL-GalK (Table 1). Primers used for deletion mutagenesis are listed in Supplementary Table 1. The strategy used to generate the *tmytC* mutant involved amplifying the gene and

its upstream and downstream regions from *T. pulmonis*-PW1004 genomic DNA, purifying the PCR product, and cloning it into pCR-XL-TOPO to generate pCR-XL-*tmytC*. The 5' and 3' flanking regions of *tmytC* were amplified from plasmid DNA of pCR-XL-*tmytC* and combined through overlapping PCR to create an in-frame deletion pattern. This pattern was then cloned into p2NIL-GalK, resulting in the final construct p Δ *tmytC*. The construct was introduced into *Escherichia coli* DH5 α by electroporation. Transformants were selected on BHI agar with kanamycin, followed by culturing on BHI agar without antibiotics and selection on BHI agar with 2-deoxy-D-galactose [29]. The resulting 2-deoxy-D-galactose-resistant colonies were assessed for kanamycin sensitivity and screened using primers *tmytC*-UF-1/DR-1. Similar strategies were used to generate deletion mutants of *tmytA* and *tmytB*. Finally, all mutants were confirmed by DNA sequencing with inner-*tmytC*-F/R primers and the expression level of mycolyltransferase genes in the wild-type (WT) *T. pulmonis* PW1004 and the three knockout mutants were determined by qRT-PCR.

Complementation of PW1004 Δ *tmytC*

The coding region of *tmytC* gene, together with its ribosome-binding site, was amplified from chromosomal DNA of PW1004 using primer *hsp60*-F/R and *tmytC*-F/R and subcloned into expression shuttle vector pNV18 [30], resulting in the final construct pNV18-tMytC. The complementation plasmid was transformed into PW1004 Δ *tmytC*, and TmytC protein was constitutively expressed. A mutant strain transformed with empty plasmid pNV18 was used as a negative control.

Biofilm growth condition and quantitation

Biofilm was formed in modified M63 medium with slight modifications [31]. *Tsukamurella* strains cultured in BHI broth at 37°C for 2 days. Saturated bacteria (10^8 CFU/ml) was then inoculated at 1:1000 in modified M63 medium in a 6-well flat bottom polystyrene plate in triplicate and incubated at 25°C for 14 days without disturbance. Biofilms were quantified using a crystal violet assay. Experiments were performed in triplicate and repeated three times.

Microscopy analysis of the biofilms

For scanning electron microscopy (SEM) analysis, biofilm was fixed in 6% glutaraldehyde for 72 h and observed using LEO 1530 FEC SEM (Zeiss, Germany). Additionally, the biofilm was stained with SYTO 9 green fluorescent nucleic acid stain and visualized using a Perkin Elmer UltraVIEW VOX Spinning

Disc confocal laser scanning microscope with 3-dimensional reconstructions created using Volocity 6.3 software.

Hydrophobicity assay

The hydrophobicity assay followed the method by Nguyen et al [32]. Droplets of oil and trypan blue (0.05% solution in sterile water) were applied to the *T. pulmonis* and its mutant biofilms cultured for 14 days in polystyrene plates. The appearance of the droplets (beading or spreading) was observed and photographed to determine the hydrophobicity of the strains.

Biofilm formation on contact lens and quantification

Senofilcon A soft contact lenses (Johnson & Johnson vision care Inc., Jacksonville, FL, USA) were used in the study. Biofilm quantification followed the method by Szczotka-Flynn et al. with slight modifications [33]. Contact lenses were washed with 1 \times PBS and incubated in cell suspensions (absorbance of 0.5 at OD₆₀₀ of the PW1004-WT and its derivative mutants) for 3 days. Lenses were then washed and immersed in 1% TSB for 3 days on a rocking platform. Biofilm was quantified by sonication and vortexing, and the number of CFUs was determined. Experiments were performed in triplicate and repeated three times.

Statistical analysis

Unless stated otherwise, data generated were expressed as mean \pm standard error of the mean (SEM) from three independent experiments. Statistical comparison between different groups was performed by the unpaired Student's *t*-test. An asterisk indicates a significant difference (*, $P < 0.05$; **, $P < 0.01$; n.s., not significant).

Results

Rabbit model of *Tsukamurella keratitis*

To understand the pathogenesis of *Tsukamurella keratitis*, we developed an eye infection model using NZW rabbits. We inoculated *T. pulmonis*-PW1004 at 10^6 CFUs/cornea by direct intrastromal injection and the pathological changes of the infected rabbit eyes were monitored daily. At 24 h PI, the rabbit corneas started to develop gross pathological signs of infection, including severe iritis, ocular discharge, corneal infiltrate, corneal erosions and dense corneal opacity, with increasing severity observed over time (Figure 1a). On the other hand, we did not observe any pathological signs in control rabbits injected

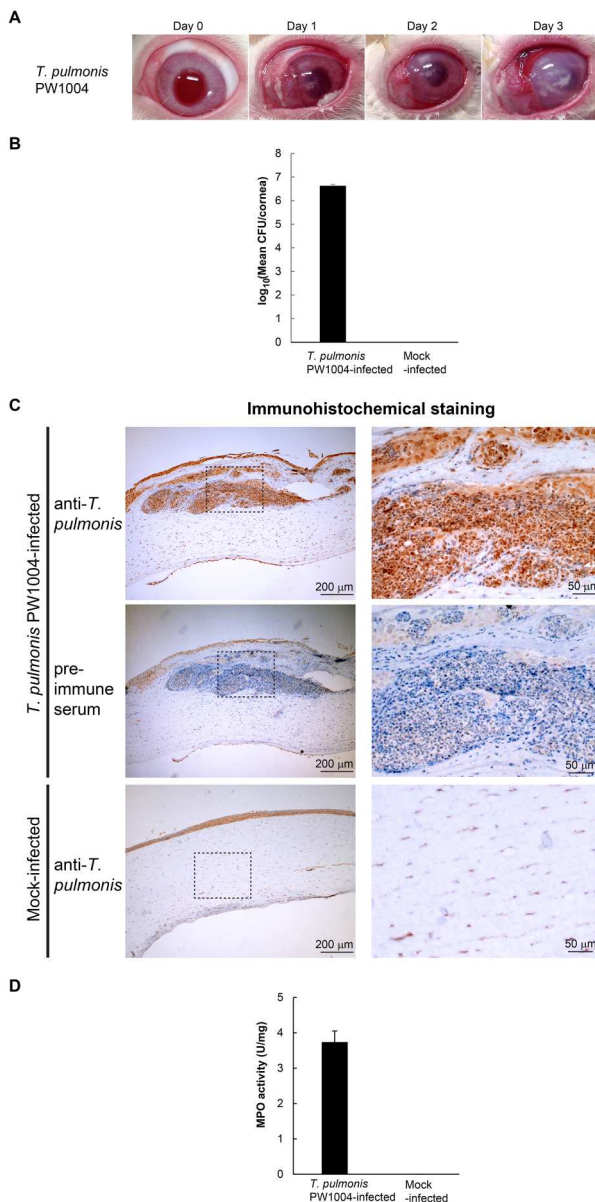


Figure 1. Experimentally induced keratitis in NZW rabbits after intrastromal injection of *T. pulmonis*-PW1004. (a) Gross appearance of the rabbit eyes after *Tsukamurella* infection. (b) Mean bacterial load recovered from the cornea of rabbits infected with *T. pulmonis*-PW1004 and those of control rabbits at 24 h PI. Error bars indicated mean CFU/cornea \pm SEM of three independent experiments. (c) Immunohistochemical staining of corneal sections using mouse anti-*T. pulmonis*-PW1004 serum. The boxed area is further enlarged and shown in the right-hand panel of the corresponding image. Strong positive staining in brown colour against *T. pulmonis* could be detected in corneal sections from rabbits infected with *T. pulmonis*-PW1004 (top) but not from the mock-infected control rabbits (bottom). The middle panel shows corneal sections from the infected rabbits stained with pre-immune control serum; corneal sections of infected rabbits showing large amount of inflammatory cell infiltration with haematoxylin counterstain (top and middle). (d) MPO activity (U/mg) of the corneal tissues harvested from rabbits. Error bars indicate mean \pm SEM of three independent experiments.

with plain culture medium only (Supplementary Figure 1). At 24 h PI, pure cultures of *Tsukamurella* at 6.64 ± 0.46 log CFU/cornea were recovered from

the infected rabbits (Figure 1b). Active infection was confirmed by the detection of *T. pulmonis*-PW1004 antigens by immunohistochemical staining using specific anti-*T. pulmonis* antibodies. Strong staining against *T. pulmonis* could be detected in corneal sections from rabbits infected with *T. pulmonis*-PW1004 (top, Figure 1c) but not from the control rabbits (bottom, Figure 1c). Specificity of the anti-*T. pulmonis* antibodies was verified by staining the corneal sections using mouse pre-immune control serum (middle, Figure 1c). Large amount of inflammatory cell infiltration was observed with the haematoxylin counterstain in the corneal sections from the *T. pulmonis*-infected rabbits (top and middle, Figure 1c). This was in line with the results of the MPO activity assay, which quantified tissue PMN accumulation. MPO activities increased to 3.87 ± 0.26 U/mg in the corneal homogenates from rabbits infected with *T. pulmonis*-PW1004 compared to those from rabbits of the control group (Figure 1d). To fulfill Koch's postulates, we used *T. pulmonis*-PW1004 recovered from the cornea of a rabbit to infect other rabbits using the same route of inoculation. Results consistently showed that the infected rabbit developed similar signs and symptoms of keratitis and histopathological changes at 24 h PI.

Genome sequencing and analysis of *T. tyrosinosolvens*-PW899 and *T. pulmonis*-PW1004

We attempted to sequence *Tsukamurella* genomes to identify potential virulence factors, particularly those related to biofilm formation, associated with *Tsukamurella*-mediated ocular infections. The draft genomes of *T. tyrosinosolvens*-PW899 and *T. pulmonis*-PW1004, two strains previously isolated from patients with ocular infections [9,10], were sequenced and assembled (Figure 2a). Sequencing generated 11–15 million clean reads per strain (estimated 410–540 \times coverage). After *de novo* assembly, the draft genome of *T. tyrosinosolvens*-PW899 was 4.88 Mb in length distributed in 307 contigs (>500 bp), and that of *T. pulmonis*-PW1004 was 4.60 Mb in length distributed in 245 contigs (>500 bp) (GenBank accession numbers of *T. tyrosinosolvens*-PW899 and *T. pulmonis*-PW1004 are QPKD00000000 and QQQF00000000, respectively) (Table 2). All contigs generated were submitted to the RAST version 2.0 annotation server, resulting in 4558 protein-coding sequences (CDSs), 3 rRNA operons and 49 tRNA-encoding genes for *T. tyrosinosolvens*-PW899 and 4241 CDSs, and 3 rRNA operons, and 47 tRNA-encoding genes for *T. pulmonis*-PW1004 (Table 2). Each CDS in the two genomes was further classified into different categories in COGs (Figure 2b) and subsystems in RAST (Figure 2c) based on their predicted

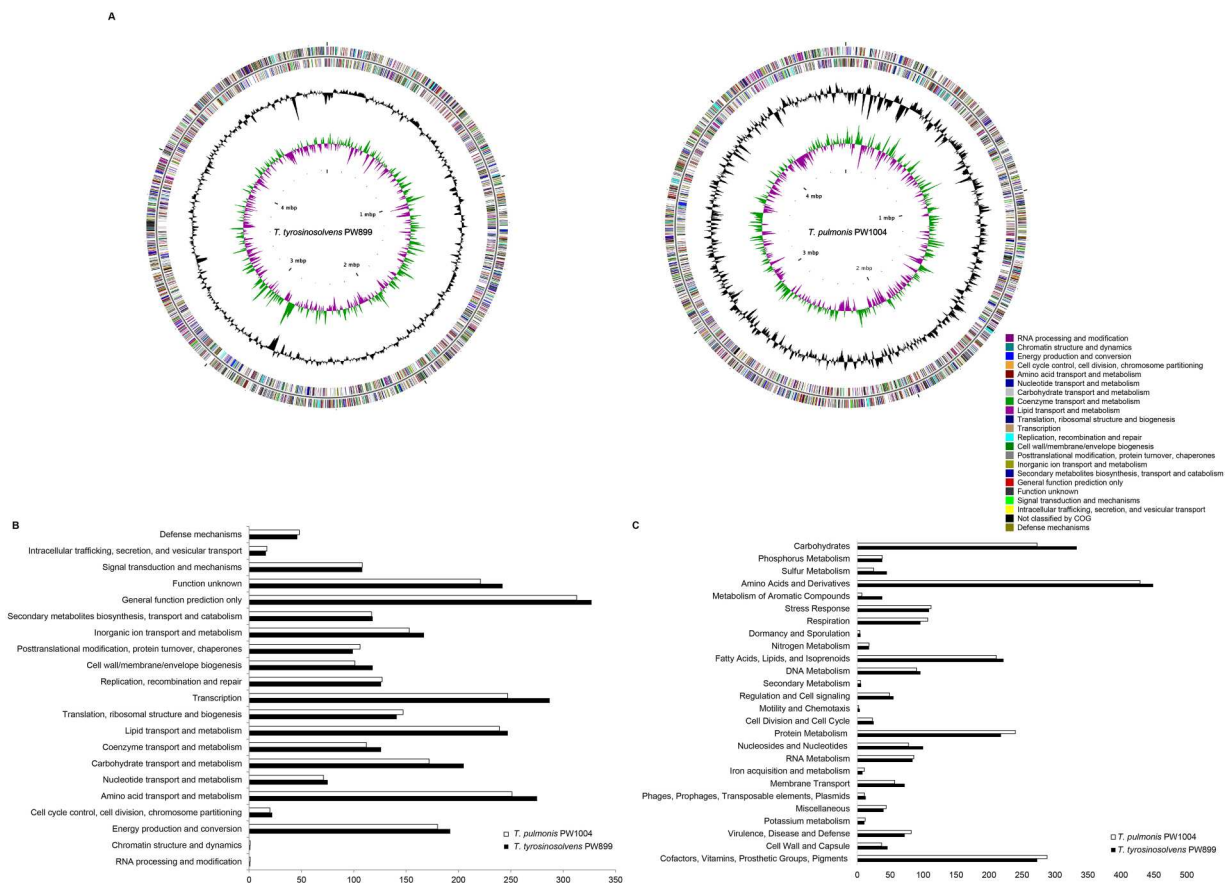


Figure 2. Graphical circular maps of the genomes and the distributions of predicted coding sequence function according to COG and SEED subsystems. (a) *T. tyrosinosolvans*-PW899 (left) *T. pulmonis*-PW1004 (right). From outside to centre, ring 1 and 2 show protein coding genes on both the forward and reverse strand (coloured by COG categories, respectively); ring 3 shows G + C% content plot, and ring 4 shows GC skew, purple indicating negative values and green, positive values; The columns indicate the number of proteins in different (b) COG and (c) SEED subsystems.

Table 2. Results of draft genome assembly of *T. tyrosinosolvans*-PW899 and *T. pulmonis*-PW1004.

Genome assembly data	<i>T. tyrosinosolvans</i> -PW899	<i>T. pulmonis</i> -PW1004
Genome size	4.88 Mb	4.60 Mb
G + C content	71.0%	70.9%
Total no. of contigs	321	266
No. of contigs (>500 bp)	307	245
No. of predicted protein-coding genes	4558	4241
No. of subsystems	399	384
No. of tRNAs	49	47
No. of rRNA operons	3	3
GenBank accession no.	QPKD00000000	QQQF00000000

functional roles. Specifically, the two *Tsukamurella* genomes contained a number of putative genes that may be involved in biofilm formation (Supplementary Table 2). Among these genes, three homologues of a gene encoding mycolyltransferase were identified in both genomes of *T. tyrosinosolvans*-PW899 and *T. pulmonis*-PW1004. Previous studies have shown that proteins which possess mycolyltransferase activities play significant roles in biofilm formation, as well as other important biological roles, in members of *Corynebacteriales* [34,35]. For this reason, we attempted to study the role of these homologues and in the pathogenesis of *Tsukamurella* keratitis.

The three mycolyltransferase-encoding homologues identified in both *Tsukamurella* genomes were designated as *tmytA* (*Tsukamurella* mycolyltransferase A), *tmytB* and *tmytC* respectively (Figure 3a). The size of the three mycolyltransferase homologues varied from 963 to 1632 bp, as a result of the lack of a C-terminal region in *tmytB* and *tmytC*. Their N-terminal regions in *T. tyrosinosolvans*-PW899 and *T. pulmonis*-PW1004 shared 38.1–68.9% and 38.9–69.6% amino acid (aa) identities respectively (Figure 3a). Phylogenetically, they were most closely related to the mycolyltransferase of *Williamsia limnetica*, also a member of *Corynebacteriales*, sharing 44–45% aa identities. Detailed annotation of the gene sequences revealed the presence of critical aa residues Ser-125, Asp/Glu-229, and His-261 in their N-terminal regions, which forms a catalytic triad (Figure 3a). This is a typical feature of other characterized mycolyltransferases and is essential for mycolyltransferase activity [35]. To further characterize the functional role of the mycolyltransferase homologues in *Tsukamurella*, individual knockout mutant strains (PW1004 Δ *tmytA*, PW1004 Δ *tmytB*, and PW1004 Δ *tmytC*) were constructed respectively in *T. pulmonis*-PW1004. Biofilm phenotypes *in vitro*

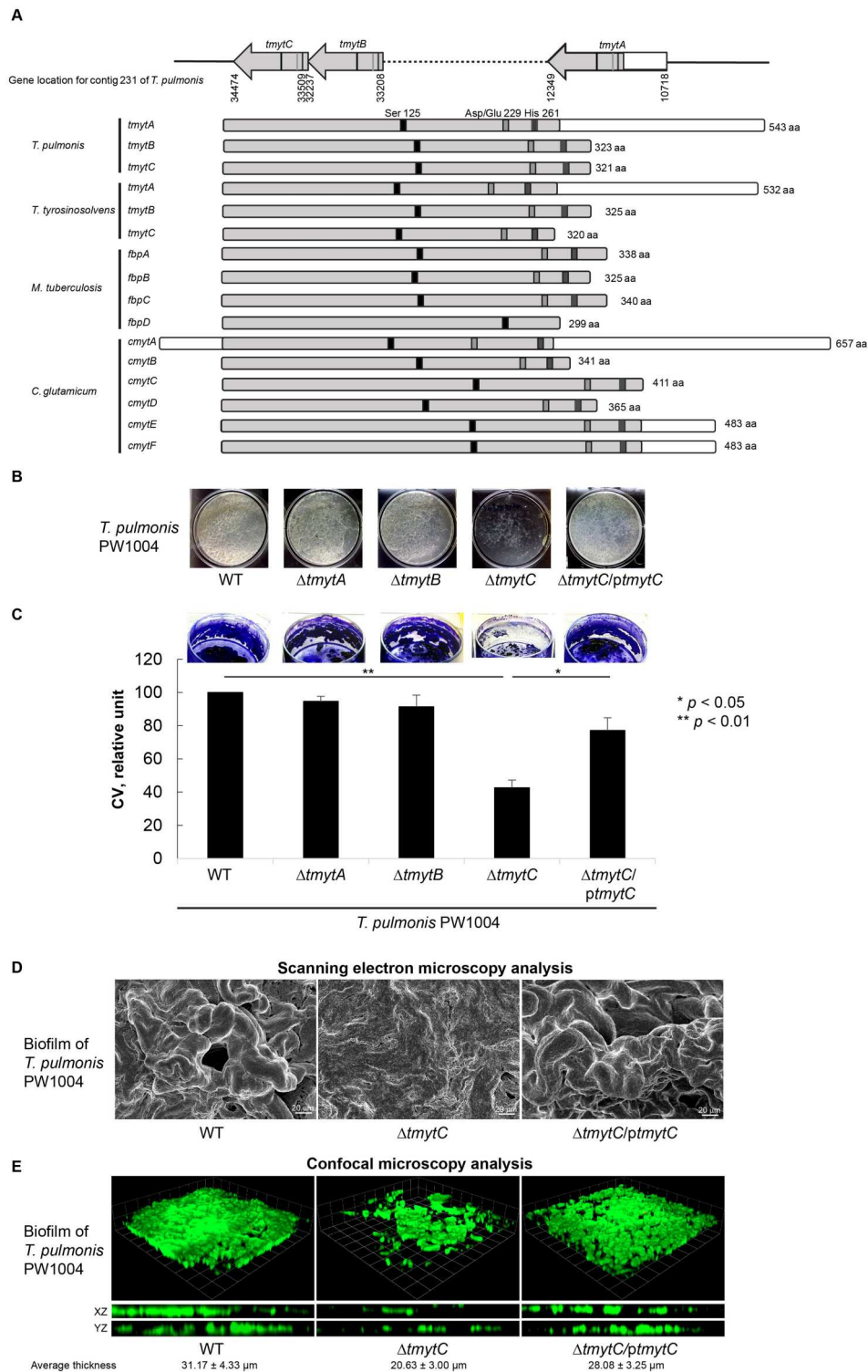


Figure 3. Characterization of the 3 *tmyt* homologues in *Tsukamurella*. (a) Locations of the *tmytA*, *tmytB*, and *tmytC* gene in the genome of *T. pulmonis*-PW1004 are indicated. Alignment of the *tmyt* homologues identified in *T. pulmonis*-PW1004, *T. tyrosinosolvens*-PW899, *M. tuberculosis* (GenBank accession numbers NP_218321, NP_216402, YP_177694 and YP_178017) and *C. glutamicum* (GenBank accession numbers AAAP23202-AAAP232007). The catalytic triad formed by functional residues Ser125, Asp/Glu229, and His261, which are important for mycolyltransferase activity, are indicated by black, grey, and dark grey boxes, respectively. (b) Biofilm formed by the PW1004-WT and its derivative mutants when they were cultured under static conditions for 3 days. With the exception of the PW1004 $\Delta tmytA$, PW1004 $\Delta tmytB$, PW1004-WT, and *tmytC* complemented mutant (PW1004 $\Delta tmytC/ptmytC$). (c) Quantitation of the biofilm formed by the PW1004-WT and its derivative mutants using crystal violet staining method. The amount of biofilms was significantly reduced in PW1004 $\Delta tmytC$ compared to the PW1004-WT ($P < 0.01$) and complemented mutant ($P < 0.05$). (d) SEM and (e) confocal microscopy analyses of *Tsukamurella* biofilms cultured under static conditions for 3 days. Representative SEM micrographs of the biofilm formed by the PW1004 $\Delta tmytC$ was flattened and less structured compared to those formed by the PW1004-WT and complemented mutant. Likewise, biofilms were fixed and stained with SYTO 9 green fluorescent stain prior to confocal microscopy analysis. Representative micrographs comparing biofilm thickness of each *Tsukamurella* strain was measured in different points of each field. The means and standard deviations of three independent experiments are shown.

and virulence *in vivo* of each mutant strain were studied and compared to those of wild-type and/or complemented strains.

Characterization of biofilm phenotypes in wild-type PW1004 and the 3 *tmyt* knockout mutants

We successfully constructed non-polar deletions of *tmytA*, *tmytB*, and *tmytC* genes in *T. pulmonis*-PW1004 and confirmed them through DNA sequencing and gene expression analysis using qRT-PCR. We hypothesized that knockout of the *tmyt* gene may impair biofilm formation in *Tsukamurella*. To investigate this, the wild-type and 3 *tmyt* knockout mutants of *T. pulmonis*-PW1004 were cultured under static conditions to allow the formation of biofilms. Results showed that dense and confluent biofilms were formed as a floating pellicle at the air-liquid interface in PW1004 Δ *tmytA*, PW1004 Δ *tmytB*, and PW1004-WT, whereas less textured and reticulated biofilms were formed in PW1004 Δ *tmytC* (Figure 3b). Biofilm quantification using the crystal violet staining method showed that the amount of biofilm was significantly reduced in PW1004 Δ *tmytC* compared to PW1004-WT ($P < 0.01$), while PW1004 Δ *tmytA* and PW1004 Δ *tmytB* produced similar levels of biofilm as PW1004-WT (Figure 3c). To rule out the possibility that knockout of *tmyt* genes may alter growth kinetics and hence biofilm formation, we measured the growth kinetics of the wild-type and knockout mutants and found that there was no significant difference between the growth rates of the wild-type and knockout mutants (Supplementary Figure 2). To confirm the importance of TmytC in *T. pulmonis* biofilm formation, we transformed a TmytC expression plasmid (pNV18-*tmytC*) into PW1004 Δ *tmytC* and studied the subsequent biofilm characteristics. Results showed that PW1004 Δ *tmytC* complemented with TmytC (PW1004 Δ *tmytC*/ptmytC) restored the biofilm phenotype with biofilm quantity levels similar to that of PW1004-WT (Figure 3b, c). Independently, we performed SEM analysis to study the biofilm structure of WT, *tmytC*-knockout, and complemented PW1004. Results showed that the biofilm formed by PW1004-WT was highly structured (left, Figure 3d). Deletion of the *tmytC* gene, however, resulted in the formation of flatter and less structured biofilms (middle, Figure 3d), whereas PW1004 Δ *tmytC*/ptmytC complemented mutant displayed similar biofilm phenotypes as PW1004-WT (right, Figure 3d). The results were further supported by confocal microscopy analysis, in which optical sectioning along the Z axis showed reduced biofilm formation with an average thickness of $20.63 \pm 3.00 \mu\text{m}$ in PW1004 Δ *tmytC* compared to those of $31.17 \pm 4.33 \mu\text{m}$ and $28.08 \pm 3.25 \mu\text{m}$ in the wild-type and complemented mutant,

respectively ($P < 0.001$), with no significant difference in the thickness of the biofilm formed between the wild-type and complemented mutant (Figure 3e). Collectively, the results supported that deletion of *tmytC* gene, but not *tmytA* or *tmytB* genes, perturbed the biofilm-forming capacity of *T. pulmonis*-PW1004, suggesting that mycolyltransferase contributes significantly to biofilm formation in *T. pulmonis*.

Reduced virulence in *tmytC* knockout mutant in rabbits

We hypothesized that biofilm formation is one of the major factors contributing to bacterial virulence in *Tsukamurella* keratitis. Hence, we examined the importance of TmytC in the pathogenesis of *Tsukamurella* keratitis *in vivo* by inoculating rabbit corneas with PW1004-WT, PW1004 Δ *tmytC*, and PW1004 Δ *tmytC*/ptmytC respectively via intrastromal injection. The keratitis symptoms in rabbits infected with PW1004 Δ *tmytC* were less severe with less purulent discharge compared to those infected with PW1004-WT and PW1004 Δ *tmytC*/ptmytC (Figure 4a). On day 2 PI, the rabbits were sacrificed and their corneas were harvested for histopathological studies and bacterial counts. Consistent with the gross appearance of the eyes (Figure 4a), histopathological examination of the corneal tissues revealed a lower degree of PMN infiltration in rabbits infected with PW1004 Δ *tmytC* as compared to PW1004-WT and PW1004 Δ *tmytC*/ptmytC, where PMN infiltration was apparent in corneal stroma and stromal oedema was prominent (Figure 4b). In addition, PW1004-WT and PW1004 Δ *tmytC*/ptmytC showed a disordered arrangement of the epithelial layer with focal loss of superficial epithelial cells, and shrinkage of the Bowman's membrane (Figure 4b). Such damages were minimally apparent in PW1004 Δ *tmytC* and absent in the normal corneal sections (Figure 4b). Moreover, the corneal bacterial loads of PW1004-WT and PW1004 Δ *tmytC*/ptmytC were significantly higher than that of PW1004 Δ *tmytC* ($P < 0.05$), which showed only about 5% recovery rate on day 2 PI (Figure 4c). Taken together, these results suggested that TmytC contributes to the pathogenesis of *Tsukamurella* keratitis, probably through enhancing the adherence of *Tsukamurella* to corneal epithelial cells *in vivo*.

Reduced bacterial adhesion on contact lens in *tmytC* knockout mutant

Adhesion ability is a key characteristic related to biofilm formation [36]. Since there was an apparent change in the biofilm phenotypes and impaired biofilm formation capacity of PW1004 Δ *tmytC* (Figure 3b-e), we hypothesized that *Tsukamurella* adherence to abiotic materials, such as contact lenses,

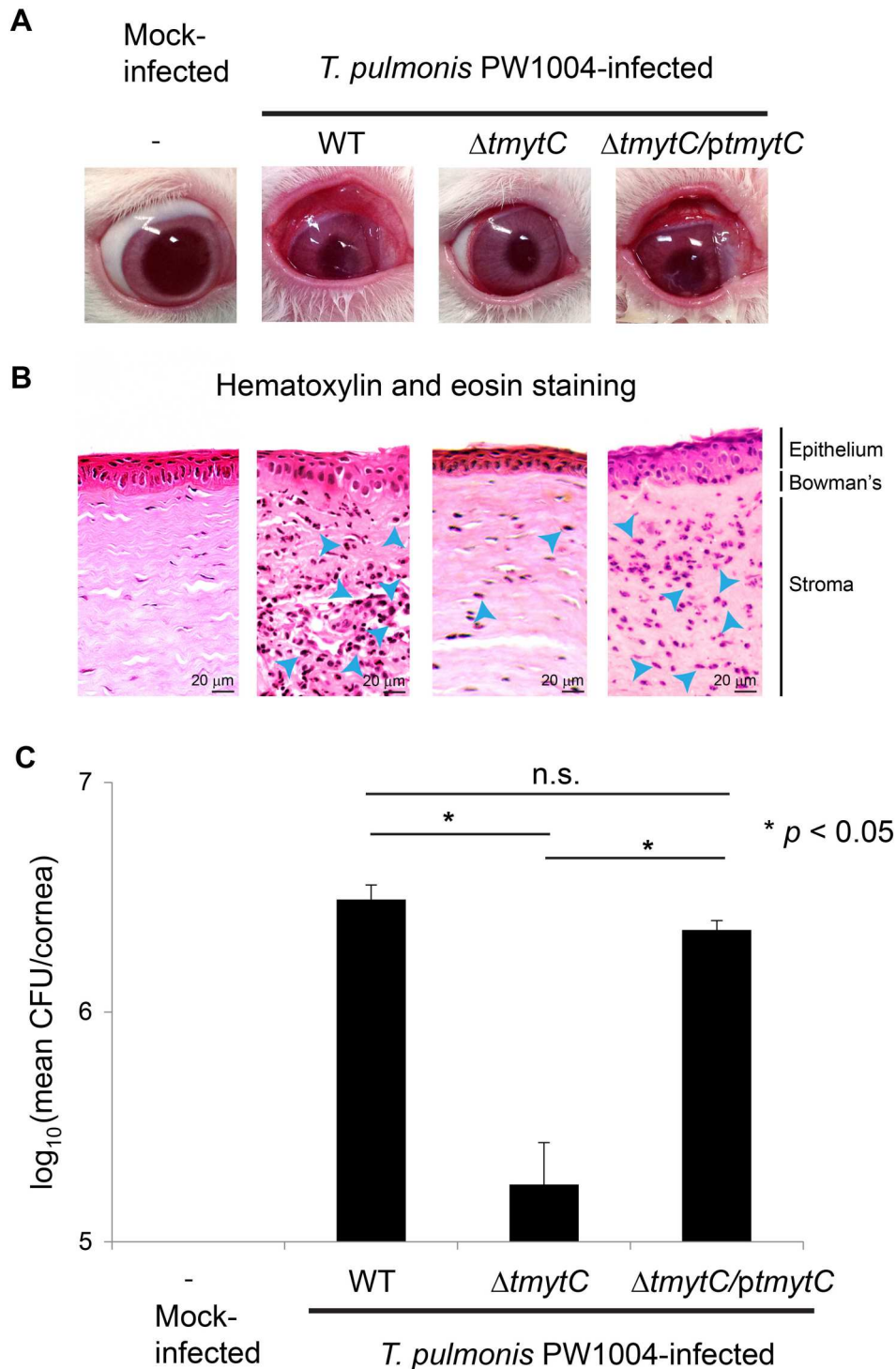


Figure 4. TmytC is a virulence factor of *T. pulmonis*. (a) Gross appearance of the rabbit eyes after intrastromal injection of the PW1004-WT and its derivative mutants. The symptoms of keratitis in rabbits infected with PW1004 $\Delta tmytC$ appeared less severe with fewer purulent discharges compared to those infected with the PW1004-WT and PW1004 $\Delta tmytC/ptmytC$ mutant. (b) Representative images of H&E staining of corneal sections of infected rabbits. PMN (shown in blue arrows) infiltration in corneal stroma and stromal oedema was less prominent in PW1004 $\Delta tmytC$ compared to the PW1004-WT and PW1004 $\Delta tmytC/ptmytC$ mutants, showing the disorderly arranged epithelial layer. (c) Mean bacterial load in cornea inoculated with the PW1004-WT, PW1004 $\Delta tmytC$, and PW1004 $\Delta tmytC/ptmytC$ at day 2 PI. Error bars indicate means \pm SEM of three independent experiments.

may also be altered. Therefore, the adhesion ability of PW1004-WT, PW1004 $\Delta tmytC$ and PW1004 $\Delta tmytC/ptmytC$ to contact lenses was determined quantitatively. Results showed that initially, all three strains were able to form a thin layer of cells over the entire surface of the contact lens. However, after subsequent mechanical detachment, washing and re-culturing, the

number of bacteria recovered from the contact lenses inoculated with PW1004 $\Delta tmytC$ (3.62 ± 0.17 log CFU) was significantly lower compared to those inoculated with PW1004-WT (5.46 ± 0.05 log CFU) and PW1004 $\Delta tmytC/ptmytC$ (5.13 ± 0.19 log CFU), respectively ($P < 0.05$) (Figure 5a). Consistently, during the culturing process of the three bacterial

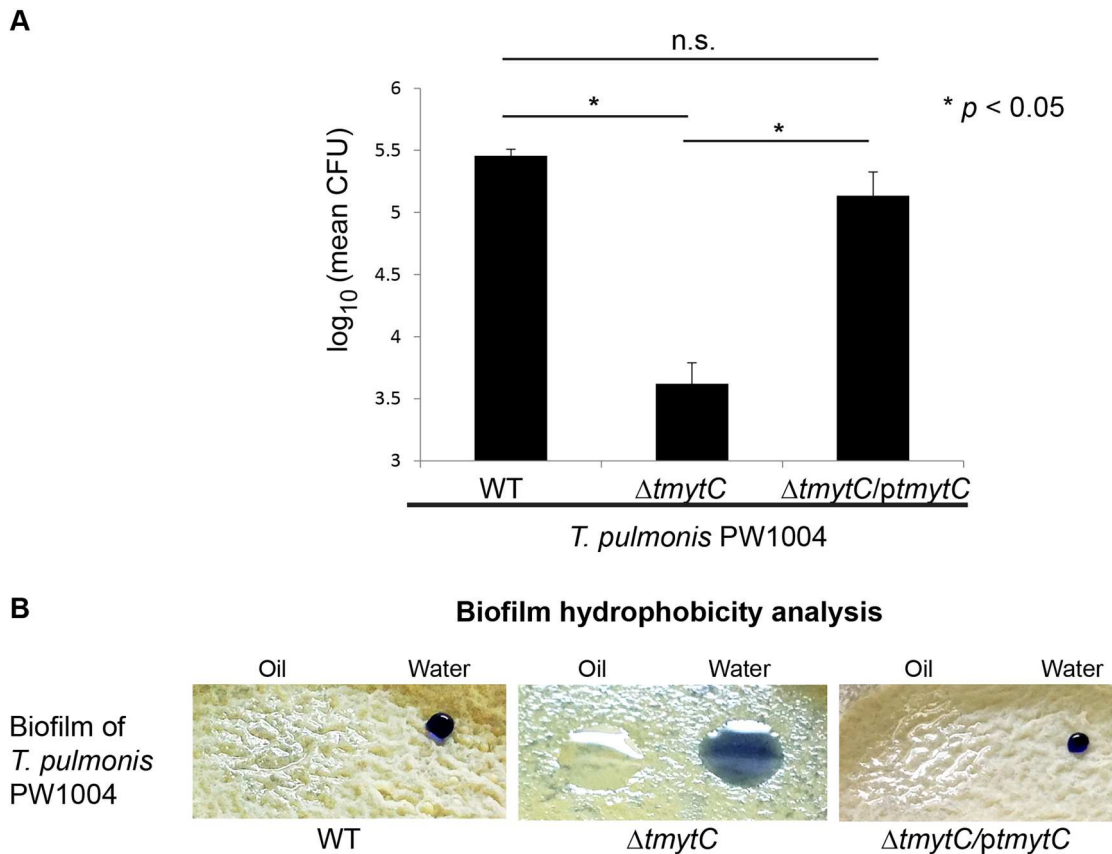


Figure 5. Altered adherence to contact lens, biofilm hydrophobicity, and PHMB susceptibility of the *tmytC* knockout mutant. (a) Adherence of the wild-type and *tmytC* mutants to contact lenses *in vitro* as analysed by CFU counting analysis. The number of bacteria recovered from the contact lenses inoculated with PW1004 $\Delta tmytC$ was significantly lower compared to those inoculated with the PW1004-WT ($P < 0.05$) and the PW1004 $\Delta tmytC/ptmytC$ ($P < 0.05$) mutants. Error bars indicated means \pm SEM of three independent experiments. (b) The PW1004 $\Delta tmytC$ surface was more hydrophilic compared to the PW1004-WT. Droplets of oil or water containing trypan blue were applied to the surface of the biofilm lawn. In the PW1004-WT and *tmytC* complemented mutant, the oil spread into a thin film over the surface, suggesting that the cell surface was hydrophobic in nature. In contrast, the water droplet continued to spread (i.e. more hydrophilic) and the oil droplet spread less (i.e. less hydrophobic) in the *tmytC* knockout mutant.

strains, a ring of cells adhering to the air-liquid interface was observed in the culture tubes of PW1004-WT and PW1004 $\Delta tmytC/ptmytC$, but not PW1004 $\Delta tmytC$ (Supplementary Figure 3). In contrast to PW1004-WT and PW1004 $\Delta tmytC/ptmytC$, a large cell pellet was observed at the bottom of the culture tube of PW1004 $\Delta tmytC$ (Supplementary Figure 3), implicating its weaker adhesion to plastic tubes. Collectively, these results demonstrated that the *tmytC* knockout mutant possessed reduced adhesion ability to contact lenses and abiotic surface (i.e. plastic culture tube), suggesting that TmytC played a role in the adherence of *Tsukamurella* to abiotic surfaces *in vitro*.

Decreased cell surface hydrophobicity in the *tmytC* knockout mutant

In addition to adhesion ability, cell surface hydrophobicity is another characteristic related to biofilm formation [37]. The reduced adhesion ability previously observed for the PW1004 $\Delta tmytC$ mutant (Figure 5a; Supplementary Figure 3) prompted us to study the

cell surface hydrophobicity in the wild-type and mutants using a visualization experiment as described previously [32]. This experiment involves adding a drop of water or oil onto the biofilm culture. If the culture cell surface is hydrophobic in nature, the water will form a bead and the oil will spread over the surface, as observed for the wild-type strain (left, Figure 5b). On the other hand, altered cell surface hydrophobicity was clearly shown in the PW1004 $\Delta tmytC$ biofilm, in which the drop of water spread out more (i.e. more hydrophilic), while oil spread out less (i.e. less hydrophobic) (middle, Figure 5b), compared to the wild-type. The biofilm hydrophobicity of the complemented mutant was restored to a comparable level as observed in the wild-type (right, Figure 5b). These observations indicated that TmytC also contributed to the cell surface hydrophobicity of *T. pulmonis*.

Discussion

In this study, we documented that *Tsukamurella* is an ocular pathogen by fulfilling Koch's postulates using

the NZW rabbit keratitis model. Bacterial keratitis is an ophthalmologic emergency that requires prompt diagnosis and expedient treatment so as to prevent visual loss. It is often associated with wearing contact lenses or other microtrauma, such as trichiasis, to the epithelial surface of the cornea. Among the cases of bacterial keratitis of which an aetiology can be identified, most are caused by *Staphylococcus aureus* and *Streptococcus pneumoniae*. Similar to animal studies in *S. aureus* [38], we demonstrated that the NZW rabbit is an excellent model for *Tsukamurella* keratitis. *T. pulmonis*-PW1004 was recovered in abundance and in pure culture from all rabbits infected with *Tsukamurella*, but the bacteria were not recovered in the control group (Figure 1b). The rabbits with keratitis caused by *T. pulmonis*-PW1004 produced clinical features similar to those observed in patients with ocular infections (Figure 1a). Histological examination further revealed marked pathological damage in corneal tissues of *Tsukamurella* inoculated rabbits but not in control rabbits (Figures 1c, 4b). When isolates of *T. pulmonis* recovered from rabbits were used to infect another group of healthy rabbits, it caused keratitis with the same clinical, pathological and histopathological characteristics, fulfilling the Koch's postulate. This animal model was used for the downstream pathogenesis studies.

tmytC, which encodes mycolyltransferase, is important for biofilm formation in *T. pulmonis*. Bacterial biofilms constitute a unique shield against antibiotic treatment and host immune reactions and is a crucial protective mechanism for bacteria that cause indwelling device infections [39]. As there is increasing evidence that biofilms play a significant role in ocular infections [40] and these as well as indwelling device infections constitute more than 90% of infections caused by *Tsukamurella*, we hypothesized that this group of bacteria is capable of producing biofilms. In this study, genome sequencing and annotation of the two ocular isolates, *T. pulmonis*-PW1004 and *T. tyrosinosolvens*-PW899, revealed a number of genes that may be involved in biofilm formation (Supplementary Table 2). Among these genes, both genomes contained three homologues of genes (*tmytA*, *tmytB*, and *tmytC*) encoding mycolyltransferase (Figure 3a). This enzyme functions by transferring one mycolate residue from trehalose monomycolate (TMM) to another molecule of TMM yielding trehalose 6,6'-dimycolate, which will then be processed by esterase to form free mycolic acids [41]. Mycolic acids contribute to many important biological roles in bacteria, such as being a key component of biofilms [31,34,35], maintaining cell wall structure [32], and mediating host cell immune activation [42]. A close relationship between mycolic acids and biofilm production has been reported in previous studies [34,35]. For example, impaired biofilm formation

due to the deletion of a gene encoding mycolyltransferase has been reported in *Mycobacterium smegmatis* [35]. In this bacterium, five mycolyltransferase gene homologues were identified in its genome. Deletion of *fbpA*, but not the other four homologues, showed reduced biofilm formation and level of mycolic acids [35]. Unlike *M. smegmatis*, which possesses five mycolyltransferase gene homologues, we only identified three mycolyltransferase homologues in each of the *T. pulmonis*-PW1004 and *T. tyrosinosolvens*-PW899 genomes. The size of these three mycolyltransferase homologues varied considerably due to the lack of a C-terminal region in two of them, similar to the situation in *Corynebacterium glutamicum* [43] (Figure 3a). In this study, we showed that deletion of *tmytC*, but not *tmytA* or *tmytB*, showed altered biofilm phenotype and impaired biofilm formation in *T. pulmonis*-PW1004, which was probably due to reduced mycolic acid production (Figure 3b–e). This shows that *tmytC* is important for biofilm formation in *T. pulmonis*. However, it remains unclear why disruption of *tmytC*, but not *tmytA* or *tmytB* in *Tsukamurella*, showed impaired biofilm production, although the 3 mycolyltransferase homologues possess identical essential motifs in their N-terminus (Figure 3a). It is speculated that mycolyltransferase homologues may exhibit differential substrate preference and/or spatial cellular localizations resulting in different activities [32]. Given the important biological roles of mycolic acids, further studies on deciphering the types, chemistry and structure of mycolic acids synthesized in *Tsukamurella*, and their specific roles in biofilm formation are of crucial importance to understand this emerging ocular pathogen.

TmytC is a virulence factor of *Tsukamurella*. Since *tmytC* is crucial for biofilm formation, we further examined its importance for the virulence of *T. pulmonis* using the NZW rabbit keratitis model. Our results showed that there was marked attenuation of virulence and survival when *tmytC* was deleted in *T. pulmonis*-PW1004, but these phenotypes were restored when the gene was complemented (Figure 4a–c). Furthermore, our subsequent mechanistic studies have also revealed that *tmytC* knockout mutant exhibited impaired adherence to contact lens *in vitro* (Figure 5a), and that deletion of *tmytC* led to decreased hydrophobicity in *T. pulmonis*-PW1004 biofilms (Figure 5b), in line with our previous observation that *T. pulmonis* was associated with keratitis in a contact lens wearer [9]. In general, bacteria with greater surface hydrophobicity adhere to various contact lenses in greater numbers than hydrophilic bacteria [44,45]. This has been demonstrated in *P. aeruginosa*, which is known to be highly hydrophobic, exhibited significantly greater adhesion to most contact lens types compared to other ocular pathogens that are less hydrophobic, such as

S. aureus, *S. pneumoniae*, and *Haemophilus influenzae* [44,45]. This phenomenon is in line with our present results, in which the *tmytC* knockout mutant with decreased hydrophobicity also showed lower adherence to contact lenses compared to PW1004-WT and the complemented mutant (Figure 5a, b). Further sequence analyses revealed that TmytC is present in all *Tsukamurella* species for which genome sequences are available, suggesting that TmytC is highly prevalent in *Tsukamurella* species and likely plays a crucial role in biofilm formation and virulence in these species. Taken together, the present results supported that TmytC is an important virulence factor for *Tsukamurella* keratitis, which is mediated through enhancing its adherence to abiotic (i.e. contact lenses, plastics) and biotic (i.e. corneal epithelial cells) surfaces and forming biofilm. Further studies on the development of inhibitors targeting TmytC are warranted for the treatment of *Tsukamurella* infections.

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Disclosure statement

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Author contributions





JT conceived and designed the study and contributed reagents, performed the laboratory and animal work, analysed and interpreted results and wrote the manuscript. YT performed the laboratory and animal work, analysed and interpreted results. SW contributed reagents, analysed and interpreted results. MY, CC, EC, and JF performed the laboratory work, analysed and interpreted results. JC and LX performed the animal work. RAY performed histological analyses. SL conceived and designed the study and contributed reagents, analysed and interpreted results. PW conceived and designed the study and contributed reagents, analysed and interpreted results and wrote the manuscript.

Data/code availability

This Whole Genome Shotgun project has been deposited in DDBJ/EMBL/GenBank under the accession numbers

QPKD00000000; BioSample SAMN09691326 for *T. tyrosinosolvans*-PW899 and QQQF00000000; BioSample SAMN09691327 for *T. pulmonis*-PW1004.

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