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Analysis of 16S-23S intergenic spacer regions of the rRNA operons in *Tsukamurella pulmonis*

J. E. MOORE*, J XU*† and B. C. MILLAR*

*Northern Ireland Public Health Laboratory, Department of Bacteriology, Belfast City Hospital, Lisburn Road, Belfast, Northern Ireland BT9 7AD, UK; and †Department of Pathogenic Biology, Xian Jiatong University, Xi'an, Shaanxi Province, People's Republic of China

The genus *Tsukamurella* was first described by Collins *et al*.[1] in 1988 following the reclassification and further molecular and phenotypic charactization of *Gordana aurantiaca, Rhodococcus aurantiaca* and other related organisms including *Corynebacterium paurometabola*, which were distinct from the other mycolic acid-containing actinomycetes The genus is phylogenetically related to the genera *Nocardia, Gordonia, Streptomyces, Rhodococcus, Corynebacterium* and *Mycobacterium* and taxonomically comprises of at least eight described species (www.bacterio.cict.fr) including *Tsukamurella inchonensis, T. paurometabola, T. pseudospumae, T. pulmonis, T. spumae, T. strandjordii, T. tyrosinosolvens* and *T. wratislaviensis.*

Tsukamurella infections have emerged over the last decade as a rare but significant cause of serious infection in immunocompromised individuals. For a comprehensive review of these cases see Schwartz *et al*.[2] Further to this review, an additional case of line-related sepsis has been shown,[3] as well as three cases of *Tsukamurella* conjunctivitis, which were treated successfully after 10 days with polymyxin B–neomycin or chloramphenicol eyedrops.[4] Concensus opinion from published reports indicates that underlying serious disease, including haematological malignancy where the patient is immunocompromised, combined with the use of indwelling catheters are important risk factors for infection with this genus.[5] Therefore, such patients are susceptible populations for *Tsukamurella* infection.

Correspondence to Dr. John E. Moore
Email: jemoore@niphl.dnet.co.uk

Little information is available for the 16S-23S intergenic spacer regions of the ribosomal RNA (rRNA) operons of members of *Tsukamurella* species. Therefore, this study aims to examine this region in a wild type isolated from a patient.

An isolate of *T. pulmonis* was isolated from the sputum of a female patient with suspected tuberculosis. The isolate was purified on Colombia agar base (CM331, Oxoid, Basingstoke, UK) supplemented with defibrinated horse blood 5% (v/v; Oxoid) and incubated at 37^{0}C for 48 h. Genomic DNA was extracted from a single colony with the Roche High Purity PCR Template kit (Roche Diagnostics, UK), in accordance with the manufacturer's instructions.

All reaction mixes were set up in a PCR hood in a separate room from that used to extract DNA and the amplification and post-PCR room, in order to minimise contamination in accordance with the laboratory guidelines of Millar *et al*.[6] Reaction mixes (50 μL) contained10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 2.5 mmol/L $MgCl_2$, 200 μmol/L (each) dATP, dCTP, dGTP and dTTP, 1.25 units *Thermus aquaticus* (*Taq*) DNA polymerase (Amplitaq, Perkin Elmer), 0.2 μmol/L (each) 16S-23S rRNA region primers (forward primer P11P[7] [5′-GAG GAA GGT GGG GAT GAC GT-3']; reverse primer 23S [5′- CCA AGG GCA TCC ACC CGT –3′]), together with 4 μL DNA template containing approximately 50 ng DNA/mL extract.

Following a 'hot start', the reaction mixtures were subjected to the following thermal cycling parameters in a Perkin Elmer 2400 thermocycler: 96^{0}C for 3 min followed by 40 cycles of 96^{0}C for 1 min, 55^{0}C for 1min, 72^{0}C for 1 min, and a final extension at 72^{0}C for 10 min. During each run, molecular grade water (Biowhittaker, Maryland, USA) instead of DNA was included randomly as a negative control, and *Staphylococcus aureus* DNA was included as a positive control.

Following amplification, samples (15 μL) were removed, electrophoresed (80V, 45 min) in 2% agarose gel (w/v; Gibco, Paisley, UK) in TAE buffer (40 mmol/L Tris, 20 mmol/L acetic acid, 1 mmol/L EDTA [pH 8.3]) and stained with ethidium bromide (5 μg/100 mL). Gels were visualised under ultraviolet (UV) illumination with a gel image analysis system (UVP Products, England) and all images were archived as digital (*.bmp) graphic files.

Amplicons for sequencing were purified with a QIAquick PCR purification kit (Qiagen, UK) and eluted in Tris-HCl (10 mmol/L, pH 8.5) prior to sequencing. Cy-5′-labelled primers (P11P and 23S) were prepared and used for sequencing in the forward and reverse direction. Amplicons were sequenced on the ALF Express II (Amersham-Pharmacia, Buckinghamshire, England) using the Thermo Sequenase fluorescence-labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech, UK; RPN 2438): 96^{0}C for 1 min, followed by 25 cycles of 96^{0}C for 10 sec, 50^{0}C for 5 sec, 60^{0}C for 5 sec, followed by a 40C hold. The resulting sequences from both reactions were aligned and compared with those stored in GenBank using the BLASTn alignment software (www.blast.genome.ad.jp/). Sequence homology identity was determined in accordance with criteria described previously.[8]

Polymerase chain reaction amplification of the isolate with the primer pair P11P/23S gave an amplicon of approximately 2000 bp, encompassing the complete 16S rRNA gene (1499

Table 1. Comparison of levels of rRNA similarity between *Tsukamurella pulmonis* and related taxa.

16S rRNA similarity (%)[9]	16S-23S intergenic spacer region (ISR) similarity*		
	Organism	Number of bases matching (% similarity)	GenBank accession number
Tsukamurella inchonensis (99.4)	*Tsukamurella pulmonis*	437/442 (98%)	AF536517
Tsukamurella paurometabola (99.3)	*Tsukamurella pulmonis*	437/442 (98%)	AF536515
Gordona bronchialis (93.2)	*Tsukamurella pulmonis*	436/442 (98%)	AF536516
Gordona rubropertincta (99.3)	*Tsukamurella pulmonis*	436/442 (98%)	AF536514
Gordona sputi (93.0)	*Corynebacterium*	41/.43 (95%)	AF536500
Gordona amarae (94.1)	*argentoratense*		
Rhodococcus rhodochrous (94.1)			
Rhodococcus globerulus (94.8)	*Nocardia*	41/44 (95%)	AF636414
Rhodococcus equi (94.8)	*corynebacterium*		
Nocardia asteroides (94.4)			
Nocardia otitidiscaviarum (94.4)	*Rhodococcus* sp.	38/40 (95%)	AF265258
Mycobacterium aichiense (94.2)			
Mycobacterium tuberculosis (92.4)	*Nocardia* sp.	37/39 (94%)	AF536477
Mycobacterium terrae (93.7)			
D. maris (95.2)			
Corynebacterium xerosis (92.2)			
Corynebacterium glutamicum (90.4)			

*present study.

bp; position 1–1499), the complete 16S-23S intergenic spacer region (441 bp; position 1500–1941) and partial 23S rRNA gene operons (9 bp; position 1942–1951). The resulting sequence was confirmed by sequencing the forward and reverse direction, and has been deposited in GenBank as *T. pulmonis* with accession number AY741505. BLASTn analysis of the 16S rRNA operon (base 1–1499) (www.ncbi.nlm.nih.gov/blast/Blast.cgi) of the isolate confirmed it as *T. pulmonis* (99% identity).

Previously, Yassin *et al*.,[9] in examining homology of the 16S rRNA operon, demonstrated that *T. pulmonis* shares a high degree of sequence homology with other species in the genus *Tsukamurella*, as well as with other closely related members of the *Actinomycetales* (Table 1). The present study demonstrates that although there is a high degree of homology between *T. pulmonis* and the other *Tsukamurella* species (>99.3%) and with other members of the *Actinomycetales* (>90.4% to 93.2%), such homology is not shared at the 16S-23S ITS level. Although GenBank contains many entries detailing the 16S-23S ISR sequences of many members of the *Actinomycetales*, there is only a low degree of homology with *Corynebacterium, Nocardia* and *Rhodococcus*, as detailed in Table 1.

In conclusion, this study has demonstrated the heterogenous diversity of the 16-23S intergenic spacer region in *T. pulmonis*, isolated from the sputum of a female patient with a chest infection. In terms of biomedical science, such genetic diversity may be exploited in order to develop new diagnostic approaches to the detection and identification of *T. pulmonis* in patients with related infection with this organism. □

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