Tsukamurella spongiae sp. nov., a novel actinomycete isolated from a deep-water marine sponge

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A Gram-positive, rod-shaped, non-spore-forming bacterium (strain K362^T) was isolated from a deep-water marine sponge collected off the coast of Curaçao in the Netherlands Antilles. On the basis of 16S rRNA gene sequence similarities, strain K362^T was shown to belong to the genus *Tsukamurella*, being most closely related to *Tsukamurella pulmonis* (99.2 %), *Tsukamurella tyrosinosolvens* (98.9 %), *Tsukamurella strandjordii* (98.8 %), *Tsukamurella pseudospumae* (98.8 %) and *Tsukamurella spumae* (98.8 %). A combination of the substrate utilization patterns, the fatty acid and mycolic acid profiles and the DNA–DNA hybridization results supported the affiliation of strain K362^T to the genus *Tsukamurella* and enabled the genotypic and phenotypic differentiation of strain K362^T from the seven recognized *Tsukamurella* species. Strain K362^T therefore represents a novel species of the genus *Tsukamurella*, for which the name *Tsukamurella spongiae* sp. nov. is proposed. The type strain is K362^T (=DSM 44990^T=NRRL B-24467^T).

The genus *Tsukamurella* was proposed by Collins *et al.* (1988), the type species being *Tsukamurella paurometabola*. This taxon currently contains seven species (in addition to the type species) with validly published names. Species recovered from clinical specimens include *Tsukamurella inchonensis* (Yassin *et al.*, 1995), *Tsukamurella pulmonis* (Yassin *et al.*, 1996), *Tsukamurella strandjordii* (Kattar *et al.*, 2001) and *Tsukamurella tyrosinosolvens* (Yassin *et al.*, 1997). The remaining species were associated with activated sludge foaming and include *Tsukamurella pseudospumae* (Nam *et al.*, 2004) and *Tsukamurella spumae* (Nam *et al.*, 2003). These species form a distinct clade within the evolutionary radiation of the mycolate actinomycetes and share very high 16S rRNA gene sequence similarity values (Kattar *et al.*, 2001; Nam *et al.*, 2003).

Strain K362^T was isolated from a deep-water marine hexactinellid sponge with associated zoanthids collected off the coast of Curaçao (the Netherlands Antilles), at a depth of 220 m, using the Harbor Branch Oceanographic Institution's *Johnson-Sea-Link II* submersible. A small section of the sponge (approx. 15 g wet weight, including both the pinacoderm and mesohyl regions) was gently rinsed in sterile natural seawater, cut into smaller pieces and then homogenized at low speed (5000 r.p.m.) with an ethanol-sterilized high-speed homogenizer (VirTis). The sponge suspension was heat-treated (70 °C for 15 min) and plated onto maltose-seawater agar (Olson *et al.*, 2000). Strain K362^T was isolated after 28 days incubation in the dark at ambient temperature (20–25 °C). Colonies were transferred to fresh plates of the isolation media and ultimately maintained on slants of marine agar 2216 (Becton Dickinson). Morphological observations were made with a light microscope (BH-2; Olympus), using cultures grown in marine broth [5 g peptone, 1 g yeast extract, 1 ml trace metal solution (Olson *et al.*, 2000), 1 l artificial seawater (Sieburth, 1979)].

Tolerance of various temperatures (10, 25, 30, 37, 46 and 55 °C) was tested using marine agar. Tolerance of salt (NaCl at 0, 0.5, 1.0, 2.0, 3.0 and 4.0 %, w/v; KCl at 0, 1.0, 2.0, 3.0 and 4.0 %, w/v) was determined using modified marine broth (5 g peptone, 1 g yeast extract, 1 ml trace metal solution, 1 l distilled H_2O) at 22 °C with agitation.

The ability of the organism to grow on a range of sole carbon sources was examined using the basal medium of Boiron *et al.* (1993) and the methods employed by Nam *et al.* (2003, 2004). Fatty acid and mycolic acid analyses were performed on a fee-for-service basis by Microbial ID, Inc. (USA), using

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain K362^T is AY714239.

The fatty acid profiles of strain K362^T and other species of the genus *Tsukamurella* are presented in a supplementary table with the online version of this paper.

strain K362^T grown on nutrient agar at room temperature for 48–72 h.

Extraction of genomic DNA and PCR amplification using universal bacterial primers 8F and 1492R were performed as described by Olson *et al.* (2002). Amplified fragments were ligated into plasmid pCR 2.1 (TA cloning kit; Invitrogen) and used to transform *Escherichia coli* according to the manufacturer's instructions. Plasmids with inserts of the correct size were sequenced at the Macrogen (Korea) sequencing facility.

Total genomic DNA from strains K362^T, *T. pulmonis* IMMIB D-1321^T and *T. strandjordii* BAA-173^T was purified using the procedure described by Ausubel *et al.* (1987). Genomic DNA (500 μg) from each strain was sonicated to generate DNA fragments of 400–600 bp. The concentration and purity of the resulting DNA were determined from the A_{260} and the A_{260}/A_{280} ratio.

Melting temperatures (T_m) were determined using previously described procedures (De Ley *et al.*, 1970; Gillis *et al.*, 1970). DNA (30 μg) from each of the three strains was denatured in 16× SSC buffer (pH 7.0) by increasing the temperature of the sample from 32 to 98 °C (at a rate of 0.7–1.0 °C min^{-1}); optical density measurements were then recorded at 260 nm. The melting-temperature analysis was confirmed in 16× SSC buffer (pH 7.0) by using SYBR Green I (Roche Applied Science) double-stranded DNA-binding dye and a Cepheid Smart Cycler instrument (Panicker *et al.*, 2004). The T_m values for each DNA sample were recorded from the fluorescent readings obtained with the SYBR Green I dye following 50 % dissociation of the DNA. The DNA G+C content was determined by using the equation of Xu *et al.* (2000) with the value for G+C content (mol%) for *T. pulmonis* as reported by Yassin *et al.* (1996).

DNA–DNA hybridization was performed by using DNA reassociation kinetics as described previously (De Ley *et al.*, 1970; Johnson, 1985). Purified, sonicated genomic DNA (65 μg) from each strain was used according to the protocol of Pikuta *et al.* (2005).

On marine agar, strain K362^T formed dry, matte, cream-coloured colonies with irregular spreading margins and raised, wrinkled, rough centres. Wet mounts prepared from cells grown in marine broth showed large clusters of non-motile short rods (1 × 2–5 μm). Strain K362^T showed growth at 0–4 % KCl and NaCl (w/v), the optimum salt concentration being 0–1 % (w/v).

A comparison of substrate utilization patterns showed strain K362^T to be distinct from the recognized species of the genus *Tsukamurella*. Some characteristic and differential properties of *Tsukamurella* species are given in Table 1. The fatty acid composition of strain K362^T was found to be typical of those of members of the genus *Tsukamurella* (see the comparison with other species presented in Supplementary Table S1, available in IJSEM Online) and was composed of

Table 1. Phenotypic properties that distinguish strain K362^T from the type strains of species of the genus *Tsukamurella*

Strains: 1, strain K362^T; 2, *T. pseudospumae* N1176^T; 3, *T. inchonensis* IMMIB D-771^T; 4, *T. paurometabola* DSM 20162^T; 5, *T. pulmonis* IMMIB D-1321^T; 6, *T. spumae* N1171^T; 7, *T. strandjordii* ATCC BAA-173^T; 8, *T. tyrosinosolvens* IMMIB D-1397^T. Data for recognized species are taken from Nam *et al.* (2003, 2004). +, Positive; −, negative.

Characteristic	1	2	3	4	5	6	7	8
Colony colour	White, cream	Orange, red	White, cream	White, cream	White, cream	Orange, red	White, cream	White, cream
Growth at 10 °C	−	+	−	+	−	−	−	−
Growth on sole carbon sources:								
(+)-D-Arabinose	−	+	+	−	+	−	−	+
(+)-L-Arabinose	+	+	−	−	+	+	−	+
(+)-D-Arabitol	+	+	−	−	+	+	+	+
(+)-D-Cellobiose	+	−	−	−	+	−	−	+
Dulcitol	−	−	−	−	+	+	−	+
meso-Erythritol	−	−	−	−	+	+	−	+
(+)-D-Fructose	+	+	−	+	+	+	−	+
(+)-D-Maltose	−	+	+	−	+	+	−	+
(−)-D-Mannitol	+	−	+	+	−	+	+	+
(+)-D-Melezitose	+	+	+	−	−	+	−	+
(+)-D-Melibiose	+	−	+	+	+	+	+	+
(−)-D-Ribose	+	+	+	+	+	+	−	+
(+)-D-Salicin	+	−	+	+	+	−	+	+
(−)-D-Sorbitol	+	−	+	−	−	+	+	+
(+)-D-Xylose	+	−	+	+	+	+	−	+

$C_{18:1}\omega9c$ (40.7 %), $C_{16:0}$ (27.7 %), summed feature 3 ($C_{16:1}\omega7c$ and/or C_{15} iso 2-OH; 10.3 %), $C_{20:1}\omega9c$ (8.5 %), $C_{14:0}$ (3.8 %), $C_{18:0}$ (3.0 %), tuberculostearic acid (1.5 %), $C_{20:0}$ (1.1 %), $C_{17:0}$ (0.86 %), summed feature 6/7 ($C_{19:1}\omega9c$ and/or $C_{19:1}\omega11c$; 0.70 %), $C_{17:1}\omega8c$ (0.66 %), $C_{15:0}$ (0.56 %), $C_{16:1}\omega9c$ (0.54 %) and $C_{12:0}$ (0.25 %). Analysis of the mycolic acids indicated that those present in strain K362^T (number of carbon atoms: 58–75) were also typical of those present in other tsukamurellae and that the size range of the mycolic acids was representative of that of recognized *Tsukamurella* strains (64–78 carbon atoms; Collins *et al.*, 1988).

The 16S rRNA gene sequence of strain K362^T was a continuous stretch of 1480 bp. The levels of sequence similarity with respect to other tsukamurellae in GenBank were as follows: 99.2 % (over 1464 bases) for *T. pulmonis*, 98.9 % (over 1480 bases) for *T. tyrosinosolvens* and 98.8 % for *T. strandjordii*, *T. pseudospumae* and *T. spumae*.

The T_m of the genomic DNA of strain K362^T was 75 ± 0.85 °C (mean ± SD, $n=3$), whereas it was 73 ± 0.3 °C for *T. pulmonis* IMMIB D-1321^T and 62 ± 1.6 °C for *T. strandjordii* BAA-173^T. No significant differences in the T_m values determined with the spectrophotometer and the Cepheid Smart Cycler were noticed. The G + C content of the DNA of strain K362^T is 74.6 mol%.

DNA–DNA hybridization between strain K362^T and closely related strains (selected on the basis of 16S rRNA gene sequence similarity) was performed. The levels of relatedness between strain K362^T and *T. pulmonis* IMMIB D-1321^T [48 ± 1.3 % (mean ± SD, $n=3$)], strain K362^T and *T. strandjordii* BAA-173^T (44 ± 1.2 %), and *T. pulmonis* IMMIB D-1321^T and *T. strandjordii* BAA-173^T (41 ± 0.8 %) indicate that strain K362^T can be considered as a novel taxon.

On the basis of phenotypic and genotypic characteristics (cellular and colonial morphology, salt tolerance, the substrate utilization pattern, the fatty acid and mycolic acid profiles, 16S rRNA gene sequence and DNA–DNA hybridization data), strain K362^T represents a novel species of the genus *Tsukamurella*, for which the name *Tsukamurella spongiae* sp. nov. is proposed.

Description of *Tsukamurella spongiae* sp. nov.

Tsukamurella spongiae (spon′gi.ae. L. gen. n. *spongiae* of a sponge, referring to the source of isolation, a deep-water sponge).

Aerobic, Gram-positive, non-motile, non-spore-forming actinomycete. Cells are straight to slightly curved rods. Growth is observed on brain-heart infusion agar, nutrient agar and marine agar after 24–48 h incubation at ambient temperature (approx. 25 °C). Optimal temperature for growth is 25–37 °C. No growth occurs at 10 °C or above 45 °C. On all media, colonies are dry, matte and cream-coloured with irregular spreading margins and raised, wrinkled, rough centres. Colonies range in size from 2 to 5 mm in diameter and show irregular elevation. Utilizes (+)-D-galactose, (+)-D-glucose, (+)-D-mannose, α-L-rhamnose, (+)-D-sucrose and (+)-D-trehalose as sole carbon sources, in addition to those listed in Table 1. Does not utilize amyl alcohol (1 %, v/v) or methanol (1 %, v/v). Tolerates NaCl concentrations up to 4 %, but growth is enhanced at lower salt concentrations. The major cellular fatty acids of the type strain are $C_{18:1}\omega9c$ (40.7 %), $C_{16:0}$ (27.7 %) and $C_{16:1}\omega7c$ and/or C_{15} iso 2-OH (10.3 %). Tuberculostearic acid (1.5 %) is also present. Contains mycolic acids with 58–75 carbon atoms.

The type strain, strain K362^T (= DSM 44990^T = NRRL B-24467^T), was isolated from a deep-water sponge collected off the coast of Curaçao in the Netherlands Antilles, at a depth of 220 m.

Acknowledgements

We wish to thank Drs J. Euzéby and H. Trüper for assistance with nomenclature, Dr T. Neblett for isolation work, Dr R. Findlay for editorial suggestions, and the crews of the R/V *Edwin Link* and the *JSL II* submersible for sample collection. This paper is Harbor Branch Oceanographic Institution contribution number 1654.

References

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Sideman, J. G., Smith, J. A. & Struhl, K. (1987). *Current Protocols in Molecular Biology*. New York: Wiley.

Boiron, P., Provost, F. & Duport, B. (1993). *Laboratory Methods for the Diagnosis of Nocardiosis*, pp. 107–126. Paris: Institut Pasteur.

Collins, M. D., Smida, J., Dorsch, M. & Stackebrandt, E. (1988). *Tsukamurella* gen. nov., harboring *Corynebacterium paurometabolum* and *Rhodococcus aurantiacus*. *Int J Syst Bacteriol* **38**, 385–391.

De Ley, J., Cattoir, H. & Reynaerts, A. (1970). The quantitative measurements of DNA hybridization from renaturation rates. *Eur J Biochem* **12**, 133–142.

Gillis, M., De Ley, J. & De Cleene, M. (1970). The determination of molecular weight of bacterial genome DNA from renaturation rates. *Eur J Biochem* **12**, 143–153.

Johnson, J. L. (1985). DNA reassociation and RNA hybridization of bacterial nucleic acids. *Methods Microbiol* **18**, 33–74.

Kattar, M. M., Cookson, B. T., Carlson, L. D., Stiglich, S. K., Schwartz, M. A., Nguyen, T. T., Daza, R., Wallis, C. K., Yarfitz, S. L. & Coyle, M. B. (2001). *Tsukamurella strandjordae* sp. nov., a proposed new species causing sepsis. *J Clin Microbiol* **39**, 1467–1476.

Nam, S.-W., Chun, J., Kim, S., Kim, W., Zakrzewska-Czerwinska, J. & Goodfellow, M. (2003). *Tsukamurella spumae* sp. nov., a novel actinomycete associated with foaming in activated sludge plants. *Syst Appl Microbiol* **26**, 367–375.

Nam, S.-W., Kim, W., Chun, J. & Goodfellow, M. (2004). *Tsukamurella pseudospumae* sp. nov., a novel actinomycete isolated from activated sludge foam. *Int J Syst Evol Microbiol* **54**, 1209–1212.

Olson, J. B., Lord, C. C. & McCarthy, P. J. (2000). Improved recoverability of microbial colonies from marine sponge samples. *Microb Ecol* **40**, 139–147.

Olson, J. B., Harmody, D. K. & McCarthy, P. J. (2002). Alphaproteobacteria cultivated from marine sponges display branching rod morphology. *FEMS Microbiol Lett* **211**, 169–173.

Panicker, G., Vickery, M. C. & Bej, A. K. (2004). Multiplex PCR detection of clinical and environmental strains of *Vibrio vulnificus* in shellfish. *Can J Microbiol* **50**, 911–922.

Pikuta, E. V., Marsic, D., Bej, A., Tang, J., Krader, P. & Hoover, R. B. (2005). *Carnobacterium pleistocenium* sp. nov., a novel psychrotolerant, facultative anaerobe isolated from permafrost of the Fox Tunnel in Alaska. *Int J Syst Evol Microbiol* **55**, 473–478.

Sieburth, J. M. (1979). In *Sea Microbes*, p. 125. Oxford University Press: New York.

Xu, H.-X., Kawamura, Y., Li, N., Zhao, L., Li, T.-M., Li, Z.-Y., Shu, S. & Ezaki, T. (2000). A rapid method for determining the G+C content of bacterial chromosomes by monitoring fluorescence intensity during DNA denaturation in a capillary tube. *Int J Syst Evol Microbiol* **50**, 1463–1469.

Yassin, A. F., Rainey, F. A., Brzezinka, H., Burghardt, J., Lee, H. J. & Schaal, K. P. (1995). *Tsukamurella inchonensis* sp. nov. *Int J Syst Bacteriol* **45**, 522–527.

Yassin, A. F., Rainey, F. A., Brzezinka, H., Burghardt, J., Rifai, M., Seifert, P., Feldmann, K. & Schaal, K. P. (1996). *Tsukamurella pulmonis* sp. nov. *Int J Syst Bacteriol* **46**, 429–436.

Yassin, A. F., Rainey, F. A., Burghardt, J., Brzezinka, H., Schmitt, S., Seifert, P., Zimmermann, O., Mauch, H., Gierth, D. & other authors (1997). *Tsukamurella tyrosinosolvens* sp. nov. *Int J Syst Bacteriol* **47**, 607–614.