Aureispira marina gen. nov., sp. nov., a gliding, arachidonic acid-containing bacterium isolated from the southern coastline of Thailand

Shoichi Hosoya,¹ Vullapa Arunpairojana,² Chatrudee Suwannachart,² Akkharawit Kanjana-Opas³ and Akira Yokota¹

Correspondence

Shoichi Hosoya

aa37106@mail.ecc.u-tokyo.ac.jp

¹Institute of Molecular and Cellular Biosciences, The University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113-0032, Japan

²Thailand Institute of Scientific and Technological Research (TISTR), 35 Moo3, Technopolis, Khlong 5, Khlong Luang, Pathum Thani 12120, Thailand

³Department of Industrial Biotechnology, Faculty of Agro-Industry, Prince of Songkla University, Hat Yai 90112, Thailand

Three strains of gliding bacteria, 24^T, 62 and 71, isolated from a marine sponge and algae from the southern coastline of Thailand, were studied using a polyphasic approach to clarify their taxonomic positions. A phylogenetic analysis based on 16S rRNA gene sequences showed that the three isolates formed a distinct lineage within the family 'Saprospiraceae' of the phylum *Bacteroidetes* and were related to members of the genus *Saprospira*. The G + C contents of the isolates were in the range 38–39 mol%. The major respiratory quinone was MK-7. The predominant cellular fatty acids were 20 : 4 ω 6c (arachidonic acid), 16 : 0 and iso-17 : 0. On the basis of morphological, physiological and chemotaxonomic characteristics, together with DNA–DNA hybridization data and 16S rRNA gene sequences, the isolates represent a novel species of a novel genus, for which the name *Aureispira marina* gen. nov., sp. nov. is proposed. The type strain of *Aureispira marina* is 24^T (=IAM 15389^T = TISTR 1719^T).

The family 'Saprospiraceae' (Garrity & Holt, 2001), belonging to the phylum *Bacteroidetes*, comprises three genera: *Saprospira*, *Haliscomenobacter* and *Lewinella*. The genera *Saprospira* and *Haliscomenobacter* each accommodate one species, *Saprospira grandis* (Reichenbach, 1989) and *Haliscomenobacter hydrossis* (Mulder, 1989), and the genus *Lewinella* accommodates three species, *Lewinella cohaerens*, *Lewinella persica* and *Lewinella nigricans* (Sly *et al.*, 1998). In this study, three gliding bacterial strains belonging to the family 'Saprospiraceae' were isolated from marine sponge and algae in Thailand. On the basis of the results of polyphasic analysis, we propose that the isolates belong to a novel species of a novel genus in the family 'Saprospiraceae'.

Samples were collected from marine sponges, algae and woody materials from the southern coastline of Thailand. The SWG medium (pH 7.2) used for isolation was made from seawater (filtered through a Whatman No.1 filter paper to remove macroscopic particles) and 0.1 % sodium glutamate, solidified with 1.5 % agar (Difco).

The isolation medium was inoculated by allowing a drop (approx. 0.05 ml) of undiluted sample to flow in a narrow band (a little off-centre) across the surface of each agar plate. The plates were then freed of surface moisture by exposure to 22 °C for 10 min. Such drying minimized the tendency of flagellated organisms to spread across the surface (Sangkrobol & Skerman, 1981). The plates were incubated at 25 °C for 3–4 days and then examined by phase-contrast microscopy (10 $\times$ objective) to observe microcolony development.

Purification of the gliding bacteria was performed by using the Skerman micromanipulation technique (Skerman, 1968) as described by Sly & Arunpairojana (1987).

Strain 24^T was isolated from marine plant debris from Trang Province, strain 62 was from a marine sponge from Krabi Province and strain 71 was from a marine alga from Krabi Province. The isolates were then cultured and maintained at 25 °C on Sap2 medium [0.5 $\times$ artificial seawater (ASW) (1.5 % NaCl, 0.035 % KCl, 0.54 % MgCl₂·6H₂O, 0.27 % MgSO₄·7H₂O and 0.05 % CaCl₂·2H₂O), 0.1 % tryptone, 0.1 % yeast extract and 1.5 % agar].

To determine the phylogenetic relationships of the novel strains, the 16S rRNA gene was amplified by using the

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains 24^T, 62 and 71 are AB245933–AB245935, respectively.

method of Hiraishi (1992). PCR products were purified by using the PEG precipitation method (Kusukawa *et al.*, 1990). Amplicons were directly sequenced using the BigDye Terminator version 1.1 cycle sequencing kit (Applied Biosystems). Sequences were edited and assembled using the BioEdit program (Hall, 2004) and were compared with sequences from the GenBank database by using BLAST (Altschul *et al.*, 1990). Alignments of the 16S rRNA gene sequences of the isolates and members of phylogenetically related genera were carried out with the CLUSTAL X program (version 1.83; Thompson *et al.*, 1997). Nucleotide substitution rates (K_{nuc} ; Kimura 1980) were determined, and a distance matrix tree was constructed using the neighbour-joining method (Saitou & Nei, 1987). Alignment gaps and unidentified base positions were not taken into consideration in the calculation. The topology of the phylogenetic tree was evaluated by performing a bootstrap analysis with 1000 bootstrapped trials.

The results of the phylogenetic analysis based on 16S rRNA gene sequences are shown in Fig. 1. The three isolates fall within the family 'Saprospiraceae'. The 16S rRNA gene sequence of strain 24^T was identical to those of strains 62 and 71. To analyse the DNA relatedness, DNA–DNA hybridization was carried out at 40 °C and measured fluorometrically using the method of Ezaki *et al.* (1989). High levels of DNA–DNA relatedness (74–100 %) were found between the three strains. A BLAST search of the GenBank database and a phylogenetic analysis using the 16S rRNA gene sequence of strain 24^T revealed 86 % similarity to *S. grandis* ATCC 23119^T. Similarity values for *H. hydrossis* ATCC 27775^T and *L. cohaerens* ATCC 23123^T (the type strain of the type species of the genus *Lewinella*) were lower (78–80 %).

For determination of the G + C content, DNA was extracted by the method of Saito & Miura (1963) and was analysed by

the method of Mesbah *et al.* (1989). The DNA G + C contents of the isolated strains were in the range 38–39 mol%, whereas that of the reference strain, *S. grandis* NCIMB 1363^T, was 49.8 mol%.

Respiratory quinones were extracted from dried cells (200 mg) using chloroform/methanol (2:1) and were purified by TLC. The purified respiratory quinones were analysed by reversed-phase HPLC (Komagata & Suzuki, 1987). The major isoprenoid quinone of the isolated strains and *S. grandis* NCIMB 1363^T was MK-7.

The phenotypic characteristics of the isolates and *S. grandis* NCIMB 1363^T were determined as follows. Growth at different temperatures (8–37 °C) was tested on SP5 agar (containing 0.9 % casitone, 0.1 % yeast extract, 0.5 × ASW and 1.5 % agar). Salt tolerance was tested on R2A agar (Difco) supplemented with ASW (0–200 %, v/v). Growth at different pH values was tested on SP5 agar at pH 5–9. Oxidase and catalase activities were tested by spreading the cell pellet on oxidase test paper (Nippon Suisan Kaisha) and then putting 3 % H₂O₂ on top. The degradation of casein (Smibert & Krieg, 1994), DNA (using DNA agar from Nippon Suisan Kaisha), starch (Smibert & Krieg, 1994), Tweens 20, 40, 60 and 80 and L-tyrosine (Barrow & Feltham, 1993) was determined using media to which 0.5 × ASW had been added. The decomposition of alginate was tested using basal seawater medium (containing 0.1 % peptone, 0.1 % NH₄Cl, 0.0075 % KH₂PO₄·3H₂O, 0.0028 % FeSO₄·7H₂O, 0.61 % Tris, 2 % alginate, 1 % agar and 0.5 × ASW). The hydrolysis of agar and carboxymethyl cellulose was tested by inoculating stab cultures prepared using Sap2 medium containing 1 % agar or SP5 medium containing 3 % carboxymethyl cellulose. Acid production from carbon sources was assessed using modified O/F medium (Smibert & Krieg, 1994). Tests with the commercial systems API ZYM and API 20E (bioMérieux) were generally performed according to the manufacturer's instructions. The API ZYM tests were read after 4 h incubation at 30 °C and API 20E tests were read after 48 h at 30 °C. Cell movement at colony edges was verified by using phase-contrast microscopy. For analysis of the cellular fatty acids, cells were grown for 48 h at 25 °C on trypticase soy agar (BBL) supplemented with 0.5 × ASW and then analysed by using the GC-based Microbial Identification system (MIDI). Arachidonic acid was assayed using a Shimadzu model GCMS-QP5050 gas chromatograph/mass spectrometer with a DB-1 column (J&W Scientific) and identified by comparison with an authentic arachidonic acid standard (Sigma). The flow of helium gas was 1.0 ml min⁻¹, the column split ratio was 1:30 and the injection-port temperature was 250 °C. The mass spectrometer was run in EI mode, and the column temperature was programmed to increase from 180 to 270 °C at 8 °C min⁻¹.

Fig. 1. Neighbour-joining phylogenetic tree of novel strains 24^T, 62 and 71 and related members of the phylum *Bacteroidetes*, based on 16S rRNA gene sequence analysis. Bootstrap values greater than 500 are given at the branch-points. Bar, 0.02 K_{nuc} .

All of the strains were Gram-negative, aerobic, non-sporulating, non-fruiting, gliding bacteria. The novel isolates formed yellowish-orange colonies and the cells were 0.8–1.2 by 1.5–2.5 µm, forming flexible helical

Fig. 2. Phase-contrast micrograph of cells of strain 24^T. The strain was grown on Sap2 medium at 25 °C for 48 h. Bar, 10 µm.

filaments up to 100 µm long (helix width, 1.5–2.0 µm; helix pitch, 4–9 µm) (Fig. 2). The phenotypic characteristics are given in the species description and are shown in Table 1. The isolated strains and *S. grandis* NCIMB 1363^T have many similar characteristics, but there were differences in trypsin activity, acid phosphatase activity and naphthol-AS-BI-phosphohydrolase activity. In addition, there were large differences in the cellular fatty acid composition (Table 2). The major cellular fatty acids in strains 24^T, 62 and 71 were 20:4ω6c (arachidonic acid), 16:0 and iso-17:0. The arachidonic acid peak for the isolates was identified by retention-time comparisons against the authentic arachidonic acid standard, and resulted in a mass peak at *m/z* 318. In contrast to the isolates, the reference strain *S. grandis* NCIMB 1363^T contained iso-17:0 3-OH, iso-15:0 and iso-15:1 G.

To date, four marine arachidonic acid-producing bacteria have been isolated: *Psychroflexus torquis* (Bowman *et al.*, 1998), *Psychroflexus pacifica* (Iizuka *et al.*, 2003), *Krokinobacter eikastus* and *Krokinobacter diaphorus* (Khan *et al.*, 2006). The arachidonic acid contents for *Psychroflexus torquis* (four strains), *Psychroflexus pacifica* SIR-1^T, *K. eikastus* PMA-26^T and *K. diaphorus* MSKK-32^T were 2.1, 17.5, 5 and 8 %, respectively. By contrast, the cells of our

isolates were unusual in containing large amounts of arachidonic acid (about 40 %).

On the basis of the results described above, we propose that the isolates belong to a novel genus and species within the family ‘*Saprospiraceae*’ (phylum *Bacteroidetes*), for which the name *Aureispira marina* gen. nov., sp. nov. is proposed.

Description of *Aureispira* gen. nov.

Aureispira (Au.re.i.spi’ra. L. adj. *aureus* golden; L. n. *spira* a spiral; N.L. fem. n. *Aureispira* golden spiral).

Unbranched; cells are 0.8–1.2 × 1.5–2.5 µm, forming flexible helical filaments up to 100 µm long. Helix width is 1.5–2.0 µm; helix pitch is 4–9 µm (Fig. 2). Motile by gliding. Gram-negative. Chemo-organotrophic. Strictly aerobic. Require seawater for growth. Positive for cytochrome oxidase, catalase, alkaline phosphatase and acid phosphatase. The predominant fatty acid is 20:4ω6c (arachidonic acid); other characteristic fatty acids are 16:0 and iso-17:0. The respiratory quinone is MK-7. The genus belongs to the family ‘*Saprospiraceae*’ in the phylum *Bacteroidetes*. The type species is *Aureispira marina*.

Description of *Aureispira marina* sp. nov.

Aureispira marina (ma.ri’na. L. fem. adj. *marina* of or belonging to the sea, marine).

The main characteristics are as given for the genus. In addition, the optimal growth temperature is 25–30 °C; no growth occurs at 8 or 37 °C. The pH range for growth is 6.0–8.0. Growth occurs at concentrations of 20–150 % ASW. Positive for esterase (C4), esterase lipase (C8), leucine arylamidase and naphthol-AS-BI-phosphohydrolase and for degradation of casein, gelatin and Tweens 20, 40, 60 and 80. Tyrosine is degraded to coloured products. Does not decompose agar, alginate, carboxymethyl cellulose, citrate, DNA or starch. Does not reduce nitrate. No production of acetoin, H₂S or indole. Negative for lipase (C4), valine arylamidase, cystine arylamidase, trypsin, chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosamidase, α-mannosidase and α-fucosidase. Does not produce acid from arabinose, cellobiose, dulcitol, fructose, galactose, glucose, glycerol, inositol, lactose, maltose, mannitol, mannose,

Table 1. Characteristics that differentiate strains 24^T, 62 and 71 from *S. grandis* NCIMB 1363^T

Characteristic	Strains 24 ^T , 62 and 71	<i>S. grandis</i> NCIMB 1363 ^T
Colony colour	Yellowish orange	Reddish orange
Trypsin	–	+
Acid phosphatase	+	–
Naphthol-AS-BI-phosphohydrolase	+	–
DNA G+C content (mol%)	38–39	49.8

Table 2. Fatty acid profiles of strains 24^T, 62 and 71 and *S. grandis* NCIMB 1363^T

The presence of 20:4 ω 6c (arachidonic acid) was determined by GC/MS. —, Not detected.

Fatty acid	Strains 24 ^T , 62 and 71	<i>S. grandis</i> NCIMB 1363 ^T
iso-13:0	—	1.4
iso-15:1 G	—	10.0
iso-15:0	7.0–12.2	43.3
iso-16:1 G	—	1.1
16:0 N alcohol	0.3–3	—
iso-16:0	2.4–8.8	—
Summed feature 3*	—	4.1
16:0	9.6–16.9	—
iso-17:0	13.3–25.8	—
16:0 3-OH	0.1–5	3.8
iso-18:0	0.2–9	—
iso-17:0 3-OH	2.3–4.3	24.8
20:4 ω 6c	33.4–46.3	—
18:0 3-OH	0.2–1	—

*Summed feature 3 contains 16:1 ω 7c and/or iso-15:0 2-OH.

raffinose, rhamnose, sorbitol, sucrose, trehalose or xylose. The DNA G + C content is 38–39 mol%.

The type strain, strain 24^T (=IAM 15389^T = TISTR 1719^T), was isolated from marine plant debris from Trang Province, Thailand. Strains 62 (=IAM 15390 = TISTR 1728) (isolated from a marine sponge from Krabi Province) and 71 (=IAM 15391 = TISTR 1731) (isolated from a marine alga from Krabi Province) are reference strains.

Acknowledgements

We thank Taizo Sakata (Kagoshima University, Japan) for advice regarding the test for alginate decomposition.

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