Stappia marina sp. nov., a marine bacterium isolated from the Yellow Sea

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A Gram-negative, aerobic and halophilic bacterium designated strain mano18^T was isolated from a tidal flat area of Dae-Chun, Chung-Nam, Korea. This strain was motile by means of polar flagella, occasionally forming rosette-like aggregates, reduced nitrate to nitrite, required sodium ions for growth, exhibited catalase and oxidase activities and contained Q-10 as the major quinone and $C_{18:1}\omega7c$ as the dominant cellular fatty acid. Analysis of the 16S rRNA gene sequence revealed that this strain is affiliated with a cluster within the *Alphaproteobacteria*. Strain mano18^T synthesized bacteriochlorophyll under aerobic conditions. The 16S rRNA gene sequence similarity between strain mano18^T and the most closely related species, *Stappia aggregata* DSM 13394^T, was 98·5 %. Levels of DNA–DNA relatedness between strain mano18^T and the type strains of *S. aggregata* and *Stappia stellulata* were respectively 6·2–11·2 and 3·3–7·6 %. Strain mano18^T, like other *Stappia* strains, possesses carbon monoxide dehydrogenase genes. The results of DNA–DNA hybridization and the polyphasic data confirmed that strain mano18^T can be considered to represent a novel taxon in the genus *Stappia*. The name *Stappia marina* sp. nov. is proposed for the tidal flat isolate; the type strain is strain mano18^T (=KCTC 12288^T=DSM 17023^T).

The genus *Agrobacterium* has been reported to include terrestrial and plant-pathogenic species and marine species (Rüger & Höfle, 1992; Stapp & Knösel, 1954). In a study of marine star-shaped-aggregate-forming bacteria, Rüger & Höfle (1992) concluded that '*Agrobacterium aggregatum*' (Ahrens, 1968) was a later heterotypic synonym of *Agrobacterium stellulatum* (Stapp & Knösel, 1954), which had nomenclatural priority. Later phylogenetic studies based on 16S rRNA gene sequences suggested that the marine species of the genus *Agrobacterium* have no relation to the terrestrial *Agrobacterium* species; therefore, the taxonomic position of the marine subdivision of *Agrobacterium* was reassessed and a proposal was made to transfer two species belonging to *Agrobacterium* to a new genus as *Stappia aggregata* and *Stappia stellulata* (Uchino *et al.*, 1998). All known strains of the genus *Stappia* have been shown to oxidize carbon monoxide (CO) and to possess the gene for CO dehydrogenase (*coxL*) in a survey of the diversity of aerobic CO oxidizers (King, 2003).

Published online ahead of print on 7 October 2005 as DOI 10.1099/ijs.0.63735-0.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and form I and form II *coxL* gene sequences of strain mano18^T are respectively AY628423, AY753548 and AY753549.

Recently, a number of bacterial strains have been isolated in a tidal flat area as part of a study aimed at understanding the diversity of micro-organisms and their function in a tidal flat ecosystem. Many of them have been identified as phylogenetically novel micro-organisms (Yoon *et al.*, 2003a, b, c). In this study, we describe a *Stappia*-like strain, mano18^T, which was isolated from a tidal flat area of the Yellow Sea, Korea. The organism was considered to be *Stappia*-like on the basis of 16S rRNA gene sequence comparison. Accordingly, the aim of present work was to elucidate the taxonomic position of strain mano18^T by means of phenotypic, genetic and chemotaxonomic analyses.

Strain mano18^T was isolated from a sample of a tidal flat obtained in Dae-Chun, Chung-Nam, Korea (36° 17′ 45·2″ N 126° 31′ 9·5″ E) by using the dilution plating technique on marine agar 2216 (MA; Difco). The strain was routinely grown at 25 °C for 3 days. *S. aggregata* DSM 13394^T and *S. stellulata* DSM 5886^T, obtained from the DSMZ, were grown under the same conditions and used as reference strains. Morphology of live cells and the presence of flagella were investigated by using light microscopy (Nikon E600) and transmission electron microscopy (TEM). For TEM observation, cells from exponentially grown culture were negatively stained with 1 % (w/v) phosphotungstic acid. After air drying, the grid was examined by using a model H-7600

transmission electron microscope (Hitachi). Anaerobic growth was tested by using a BBL GasPak Pouch (Becton Dickinson) on MA supplemented with nitrate. Sodium ion requirements were determined by comparing growth on BM medium (Baumann *et al.*, 1971) and modified BM medium containing potassium ions in place of sodium ions and by investigating growth in trypticase soy broth without NaCl. Growth at various NaCl concentrations was investigated in marine broth 2216 (MB; Difco) or trypticase soy broth (Difco). API 20 NE test strips (bioMérieux) were used for analysing biochemical and physiological traits of bacterial strains and standard microbiological methods were used for studying Gram staining, motility and catalase and oxidase activities (Smibert & Krieg, 1994).

Isoprenoid quinones of strain mano18^T was extracted from 100 mg freeze-dried cells according to Collins & Jones (1981) and purified by preparative TLC (silica gel F254; Merck). The ubiquinone fraction was analysed by HPLC (Hitachi L-5000) equipped with a reversed-phase column (YMC pack ODS-AM; YMC Co.) as described by Shin *et al.* (1996). Bacterial strains grown on MA plates at 25 °C for 5 days were used for fatty acid methyl ester (FAME) analysis. FAMEs were extracted and prepared according to the standard protocol of the MIDI/Hewlett Packard Microbial Identification System (Sasser, 1990). The DNA G+C content was determined by the method of Tamaoka & Komagata (1984). Chromosomal DNA was extracted and purified according to the method described by Sambrook *et al.* (1989). DNA was hydrolysed and the resultant nucleotides were analysed by HPLC using a reversed-phase column (Supelcosil LC-18-S; Supelco). The 16S rRNA gene was amplified by PCR using two universal primers as described previously (Yoon *et al.*, 1998). Sequencing of the amplified 16S rRNA gene and phylogenetic analysis were performed as described by Yoon *et al.* (2003a). DNA–DNA hybridization was based on the method described by Kusuda *et al.* (1991) and Willcox (1996). DNA was transferred to a nylon membrane (Hybond-N$^+$; Amersham). The membrane was incubated for 1 h at 40 °C for prehybridization and then for 12 h at 40 °C for hybridization. A DIG High Prime DNA Labelling and Detection starter kit II (Roche Molecular Biochemicals) was used for the detection of DNA. After washing, the membrane was exposed to autoradiography film (Hyperfilm-ECL; Amersham) for 10 min and signal intensities were determined using the TINA 2.0 program (Lee *et al.*, 2003). The signal produced by self-hybridization was taken as 100 %, and relative intensities of genomic DNAs of other strains were determined to be the percentage similarity.

The *coxL* gene of strain mano18^T was amplified according to the method of King (2003). Two forward primers, OMPf [5′-GGCGGCTT(C/T)GG(C/G)AA(C/G)AAGGT-3′] and BMSf [5′-GGCGGCTT(C/T)GG(C/G)TC(C/G)AAGAT-3′], and a reverse primer, O/Br [5′-(C/T)TCGA(T/C)GATCATCGG-(A/G)TTGA-3′], were designed on the basis of conserved motifs of gene sequences for the large subunit of authentic CO dehydrogenases (King, 2003). Primer pairs OMPf and O/Br or BMSf and O/Br were used for amplification of the form I and form II large subunit genes of CO dehydrogenase, respectively.

The production of bacteriochlorophyll *a* (BChl *a*) by strain mano18^T was investigated by PCR amplification of a phototrophism-related gene (*pufM*). Primers pufMF (5′-CGCACCTGGACTGGAC-3′; Achenbach *et al.*, 2001) and pufMR [5′-CCAT(G/C)GTCCAGCGCCAGAA-3′; Beja *et al.*, 2002] were used for PCR amplification of the *pufLM* gene. Extracted genomic DNA of strain mano18^T was used as the PCR template. The PCR mixture contained 1·5 mM $MgCl_2$, 0·25 μM each dNTP, 5 U *Taq* DNA polymerase, 0·5 μM each primer and 10 ng template DNA in a total volume of 20 μl (Bioneer). PCR was performed in a thermocycler (iCycler; Bio-Rad) with an initial denaturation step at 95 °C for 4 min followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 47 °C for 40 s and extension at 72 °C for 1·5 min and a final extension at 72 °C for 7 min. The amplified DNA was separated by agarose gel electrophoresis (1 % agarose in 1× TAE), stained with ethidium bromide, viewed by UV illumination and photographed.

Cells of strain mano18^T were Gram-negative, regular and club shaped. They occurred singly, in irregular clusters or in star-like aggregates or rosette-like aggregates (Fig. 1). However, star-shaped aggregate formation could not always be observed. It seems to be dependent on the physiological state of the cells (Suzuki *et al.*, 2000). Strain mano18^T has been grown at 25–30 °C and needed sodium ions for growth. Growth occurs in the presence of 3–6 % (w/v) NaCl. Optimum growth of strain mano18^T occurred at 25 °C and in 3 % (w/v) NaCl. The physiological and biochemical properties of strain mano18^T are summarized in Table 1. A partial 16S rRNA gene sequence (1355 bp) was determined from strain mano18^T. The result of a BLAST search indicated that the isolate was a member of the *Alphaproteobacteria* and was closely related to several marine bacteria. The 16S

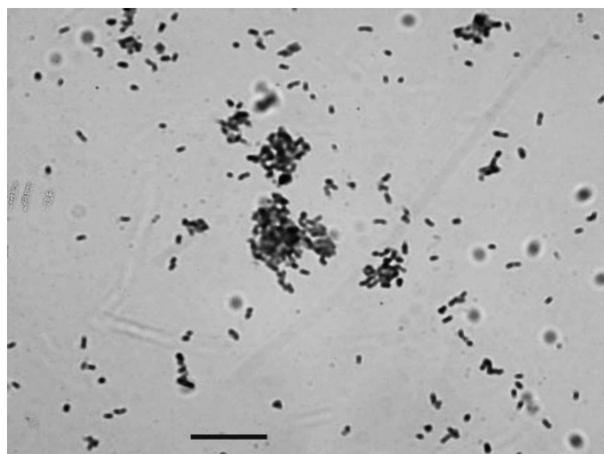

Fig. 1. Light micrograph of strain mano18^T showing rosette-like-aggregate formation. Bar, 10 μm.

Table 1. Characteristics that differentiate strain mano18^T from other *Stappia* species and *Roseibium hamelinense*

Strains: 1, *R. hamelinense* OCh 368^T; 2, mano18^T; 3, *S. aggregata* DSM 13394^T; 4, *S. stellulata* DSM 5886^T. +, Positive; −, negative; W, weakly positive; ND, no data. Data for *R. hamelinense* were taken from Suzuki *et al.* (2000). Cells of all strains are aerobic, motile rods that stain Gram-negative. All strains require NaCl and grown in 3 and 6 % NaCl. All strains reduce nitrate to nitrite. All *Stappia* strains are positive for catalase and oxidase and negative for indole production from tryptophan, arginine dihydrolase, gelatin hydrolysis and acid production from glucose. For determination of utilization of carbon sources, cultures were incubated for 7 days at 25 °C.

Characteristic	1	2	3	4
Growth in 10 % NaCl	+	W	+	W
Reduction of nitrite to N_2	−	−	+	−
Urease	−	+	+	−
Aesculin hydrolysis	ND	+	+	−
β-Galactosidase	ND	+	+	−
Utilization of carbon sources				
Glucose	+	−	+	−
Arabinose	ND	−	+	−
Mannose	ND	W	+	−
Mannitol	ND	−	+	−
N-Acetylglucosamine	ND	−	+	−
Maltose	ND	−	+	−
Gluconate	ND	−	+	−
Caprate	ND	−	−	−
Adipate	ND	−	W	W
Malate	ND	+	+	W
Citrate	ND	−	+	−
Phenylacetate	ND	−	W	−

rRNA gene sequence of strain mano18^T showed the highest similarity to that of *S. aggregata* DSM 13394^T (98·5 %); the next highest similarity was observed to members of the genus *Roseibium* (97 %) and *S. stellulata* DSM 5886^T (95 %). 16S rRNA gene sequence alignment and phylogenetic tree construction (Fig. 2) were conducted by using CLUSTAL X software (Thompson *et al.*, 1997). DNA–DNA hybridization studies were carried out between strain mano18^T and closely related strains selected on the basis of their 16S rRNA gene sequence similarities and phylogenetic positions. The low DNA–DNA relatedness between strain mano18^T and *S. aggregata* DSM 13394^T (6·2–11·2 %) and *S. stellulata* DSM 5886^T (3·3–7·6 %) confirmed that strain mano18^T represents a novel species.

Ubiquinone 10 (Q-10) was the predominant isoprenoid quinone in strain mano18^T and the G+C content of strain mano18^T was 59·7 mol%. Unsaturated fatty acids including $C_{18:1}\omega 7c$ (58·47 %) together with $C_{20:1}\omega 7c$ (7·80 %), 11-methyl $C_{18:1}\omega 7c$ (9·08 %), $C_{17:1}\omega 8c$ (0·55 %) and $C_{18:1}\omega 9c$ (0·23 %) were the most abundant. Small amounts of saturated fatty acids $C_{18:0}$ (8·03 %), $C_{19:0}$ cyclo $\omega 8c$ (3·56 %) and $C_{16:0}$ (1·65 %) were also detected. The fatty acid composition was most similar to that observed in the genus *Stappia* (Table 2) and clearly differentiated the isolate from other phylogenetically related genera (data not

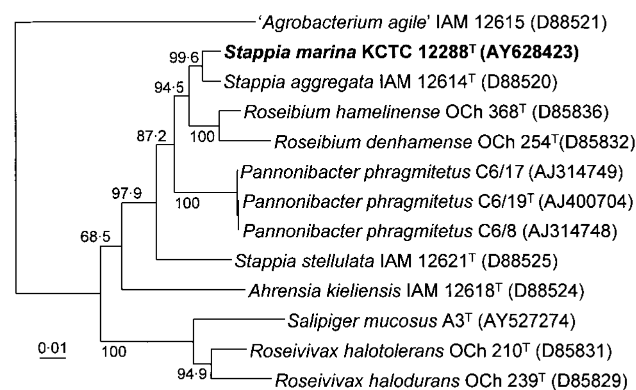

Fig. 2. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic position of strain mano18^T within closely related strains in the family *Rhodobacteraceae*. Bootstrap values (1000 replications) are shown as percentages at each node only if they are 50 % or greater. Bar, 0·01 substitutions per nucleotide position. '*Agrobacterium agile*' IAM 12615 was used as the outgroup.

Table 2. Cellular fatty acid compositions of strain mano18^T and type strains of *Stappia* species

Strains: 1, mano18^T; 2, *S. aggregata* DSM 13394^T; 3, *S. stellulata* DSM 5886^T. Values are percentages of total fatty acids; fatty acids representing less than 0·5 % in all strains were omitted. −, Not detected.

Fatty acid	1	2	3
Saturated fatty acids			
$C_{16:0}$	1·65	1·15	3·65
$C_{18:0}$	8·03	3·09	2·77
$C_{19:0}$ cyclo $\omega 8c$	3·56	−	3·22
Unsaturated fatty acids			
$C_{17:1}\omega 8c$	0·55	0·83	0·14
$C_{18:1}\omega 7c$	58·47	76·64	61·97
$C_{18:1}\omega 9c$	0·23	0·29	0·70
$C_{20:1}\omega 7c$	7·80	4·72	0·22
11-Methyl $C_{18:1}\omega 7c$	9·08	6·95	20·35
Hydroxy fatty acids			
$C_{18:0}$ 3-OH	0·75	0·55	1·47
Summed features*			
SF2	1·93	3·45	2·14
SF3	6·45	1·38	0·38
SF7	−	−	0·87

*Summed features represent groups of two or three fatty acids which could not be separated by GLC with the MIDI system. SF2, $C_{14:0}$ 3-OH/$C_{16:1}$ iso I; SF3, iso-$C_{15:0}$ 2-OH/$C_{16:1}\omega 7c$; SF7, $C_{19:1}\omega 6c$/unknown ECL 18·846.

shown). Minor quantitative differences in amounts of $C_{19:0}$ cyclo $\omega8c$ and summed feature 7 were observed between our isolate, *S. aggregata* DSM 13394[T] and *S. stellulata* DSM 5886[T]. The presence of $C_{18:1}\omega7c$ as the predominant fatty acid and Q-10 as the dominant lipoquinone are characteristics of members of the *Alphaproteobacteria* (Uchino *et al.*, 1998; Martínez-Cánovas *et al.*, 2004). The *coxL* gene was amplified and then sequenced bidirectionally. Inferred amino acid sequences derived from partial sequence of CO dehydrogenase form I (GenBank accession no. AY753548) and form II (AY753549) large subunits of strain mano18[T] were compared with corresponding sequences from the GenBank database. *coxL* sequences of strain mano18[T] showed the highest identity (95–97 %) with *S. aggregata* DSM 13394[T] and *S. stellulata* DSM 5886[T]. Strain mano18[T] synthesizes BChl *a* to grow photosynthetically under aerobic conditions, as judged by employing PCR amplification of a phototrophism-related gene (*pufM*, encoding the M subunit of the photosynthetic reaction centre, universally distributed among aerobic phototrophic bacteria; Achenbach *et al.*, 2001).

Cells of strain mano18[T] are Gram-negative rods, do not form spores and are motile by means of polar flagella. It is halophilic and requires sodium ions for growth. It is able to grow under anaerobic conditions by nitrate reduction. King (2003) showed that all known strains of *Stappia* oxidize CO and possess a gene for ribulose-1,5-bisphosphate carboxylase/oxygenase, which plays a central role in lithotrophic carbon fixation. Oxidization of CO by strain mano18[T] was not examined; however, the presence of *coxL* genes showing highest sequence similarity to those from members of the genus *Stappia* suggests that strain mano18[T] may oxidize CO, like other species in the genus *Stappia*. Therefore, on the basis of the data presented, strain mano18[T] should be placed in the genus *Stappia* as a representative of a novel species, for which the name *Stappia marina* sp. nov. is proposed.

Description of *Stappia marina* sp. nov.

Stappia marina (ma.ri′na. L. fem. adj. *marina* of the sea, marine).

Cells are Gram-negative, 1·3–2·0 µm in length and 0·6–0·8 µm in diameter, do not form spores and are motile by means of polar flagella. Cells occasionally form rosette-like aggregates. It is able to grow by nitrate reduction. Oxidase- and catalase-positive. Urease, aesculin hydrolysis and β-galactosidase are positive. Acid production from glucose, indole production from tryptophan, arginine dihydrolase and gelatin hydrolysis are negative. Halophilic; requires sodium ions for growth. Optimal NaCl concentration for growth is 3 % (w/v). Optimal growth temperature is 25 °C on MA. Nitrate is reduced to nitrite, but is not reduced to nitrogen gas. Q-10 is the predominant respiratory quinone. The principal cellular fatty acids are $C_{18:1}\omega7c$ (58·4 %), 11-methyl $C_{18:1}\omega7c$ (9·0 %), $C_{18:0}$ (8·0 %) and $C_{20:1}\omega7c$ (7·8 %). The G + C content of the DNA of the type strain is 59·7 mol%. Cells synthesize BChl under aerobic conditions.

The type strain, strain mano18[T] (= KCTC 12288[T] = DSM 17023[T]), was isolated from a sample of a tidal flat obtained in Dae-Chun, Chung-Nam, Korea.

Acknowledgements

This work was supported by grant BDM0200524, grant NNM0100512 and the KRIBB Research Initiative Program.

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