Salinispora arenicola gen. nov., sp. nov. and *Salinispora tropica* sp. nov., obligate marine actinomycetes belonging to the family *Micromonosporaceae*

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A taxonomic study was carried out to clarify the taxonomy of representatives of a group of marine actinomycetes previously designated MAR 1 and considered to belong to the family *Micromonosporaceae*. The organisms had phenotypic properties consistent with their assignment to this taxon. The strains formed a distinct taxon in the 16S rRNA *Micromonosporaceae* gene tree and shared a range of phenotypic properties that distinguished them from members of all of the genera with validly published names classified in this family. The name proposed for this novel taxon is *Salinispora* gen. nov. The genus contains two species recognized using a range of genotypic and phenotypic criteria, including comparative 16S–23S rRNA gene spacer region and DNA–DNA relatedness data. The names proposed for these taxa are *Salinispora arenicola* sp. nov., the type species, and *Salinispora tropica* sp. nov.; the type strains of these novel species have been deposited in service culture collections as strain CNH-643^T (= ATCC BAA-917^T = DSM 44819^T) and strain CNB-440^T (= ATCC BAA-916^T = DSM 44818^T), respectively.

INTRODUCTION

The family *Micromonosporaceae* Krasil'nikov 1938 emend. Stackebrandt *et al.* 1997 is a member of the suborder *Micromonosporineae*, one of the taxa which make up the order *Actinomycetales* Buchanan 1917 emend. Stackebrandt *et al.* 1997. The emended family is phylogenetically distinct but encompasses a chemotaxonomically and morphologically diverse group of filamentous organisms belonging to the genera *Actinoplanes*, *Catellatospora*, *Catenuloplanes*, *Couchioplanes*, *Dactylosporangium*, *Micromonospora* and *Pilimelia* (Koch *et al.*, 1996). The genera *Asanoa* Lee and

Hah 2002, *Longispora* Matsumoto *et al.* 2003, *Spirilliplanes* Tamura *et al.* 1997, *Verrucosipora* Rheims *et al.* 1998 and *Virgisporangium* Tamura *et al.* 2001 were subsequently added to the family. Members of all of the genera classified in the family *Micromonosporaceae* can be distinguished from one another using a combination of chemical and morphological properties (Lee & Hah, 2002; Matsumoto *et al.*, 2003).

Actinomycetes are generally considered to be indigenous to terrestrial habitats, although it is becoming increasingly apparent that they are common in marine ecosystems (Bull *et al.*, 2000; Maldonado *et al.*, 2005), as exemplified by the isolation of corynebacteria, dietziae, gordoniae, mycobacteria and rhodococci from deep-sea sediments (Colquhoun *et al.*, 1998; Takami *et al.*, 1999), and micromonosporae and streptomycetes from bathyal and coastal sediments (Goodfellow & Haynes, 1984; Jensen *et al.*, 1991; Takizawa *et al.*, 1993). There is convincing evidence that actinomycetes are adapted to marine habitats (Moran *et al.*, 1995; Mincer *et al.*, 2002), though to date only one marine genus, *Salinibacterium* Han *et al.* 2003, and three marine species, *Dietzia maris* (Nesterenko *et al.* 1982) Rainey *et al.* 1995, *Rhodococcus marinonascens* Helmke and Weyland 1984 and *Williamsia maris* Stach *et al.* 2004, have been described.

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The GenBank/EMBL/DBJ accession numbers for the 16S–23S interspacer region sequences of *Salinispora arenicola* strains CNH-643^T, CNH-646 and CNH-964 and *Salinispora tropica* strains CNB-440^T, CNB-536 and CNH-898 are AY371897, AY371898, AY371900, AY371895, AY371896 and AY371899, respectively.

However, it is evident from analyses of community DNA that current culture-based techniques grossly underestimate the diversity of actinomycetes in estuarine and marine sediments (Stach *et al.*, 2003a, b; Piza *et al.*, 2004). There is evidence that marine actinomycetes produce novel metabolites of commercial interest (Feling *et al.*, 2003; Fiedler *et al.*, 2005), as shown by the ability of a marine *Verrucosipora* strain to produce inhibitors of the *para*-aminobenzoic acid pathway (Riedlinger *et al.*, 2004).

The first conclusive evidence for the widespread and persistent occurrence of indigenous actinomycete populations in marine sediments was reported by Mincer *et al.* (2002), who isolated large numbers of strains, designated MAR 1, from geographically diverse tropical and/or subtropical locations. The strains were distinguished by morphological characteristics, small-subunit rRNA gene signature nucleotides and by an obligate requirement for sea water for growth. Phylogenetic analyses of nearly complete 16S rRNA gene sequences of seven strains showed that they formed a monophyletic clade within the family *Micromonosporaceae* that suggested novelty at the genus level. The MAR 1 isolates were provisionally assigned to a taxon informally designated '*Salinispora*' (Mincer *et al.*, 2002; Feling *et al.*, 2003).

The aim of the present study was to establish the taxonomic status of representative MAR 1 isolates by using a polyphasic approach. The resultant data show that the tested strains form a novel taxon, *Salinispora* gen. nov., which encompasses two novel species, *Salinispora arenicola* sp. nov. and *Salinispora tropica* sp. nov.

METHODS

Isolation, maintenance and cultivation. The representative MAR 1 strains (isolates CNB-440^T, CNB-536, CNH-643^T, CNH-646, CNH-898 and CNH-964) were recovered from marine sediments, as described by Mincer *et al.* (2002). The organisms were maintained on glucose/yeast/malt extract agar (ISP 2; Shirling & Gottlieb, 1966) at room temperature and as glycerol suspensions (20%, v/v) at -20 °C. Biomass for the chemical and molecular systematic studies was grown in shake flasks of ISP 2 broth (Shirling & Gottlieb, 1966) for 10 days at 28 °C, and then harvested by centrifugation. Cells for the chemosystematic studies were washed in distilled water and freeze-dried; those for the molecular systematic work were washed in NaCl/EDTA buffer (0.1 M EDTA, pH 8.0, 0.1 M NaCl) and stored at -20 °C until required. All media were prepared with artificial sea water (Aquarium Systems; <http://www.aquariumsystems.com>) to give a final concentration of 3.5% (w/v) NaCl.

Cultural and morphological studies. The cultural properties of the strains were observed on ISP 2 agar (Shirling & Gottlieb, 1966) and modified Bennett's agar (Jones, 1949) supplemented with mannitol (0.5%, w/v), soybean flour (0.5%, w/v) and NaCl (3.5%, w/v), following incubation at 28 °C for 4 weeks. Spore arrangement and spore surface ornamentation were observed by examining gold-coated, dehydrated material, prepared from 14-day-old cultures grown on modified Bennett's agar at 28 °C, using a Hitachi 5.570 scanning electron microscope. Gram (Hucker's modification; Society

of American Bacteriologists, 1957) and Ziehl-Neelsen (Gordon, 1967) preparations were examined by light microscopy following growth on ISP 2 and modified Bennett's agar plates.

Biochemical and physiological properties. The strains were examined for their ability to degrade a range of organic compounds by using established methods (Williams *et al.*, 1983) following incubation at 28 °C for 21 days, and for their capacity to grow on a range of sole carbon compounds after 14 days at 28 °C, following the procedure described by Pridham & Gottlieb (1948). Tolerance to pH, temperature and NaCl concentrations was recorded on ISP 2 agar (Shirling & Gottlieb, 1966) following growth at 28 °C for 4 weeks. Resistance to antibiotics was determined following incubation at 28 °C for 7, 14 and 21 days using ISP 2 agar (Shirling & Gottlieb, 1966) as the basal medium.

Chemotaxonomy. Standard HPLC and TLC procedures were used to determine the isomers of diaminopimelic acid (Staneck & Roberts, 1974), fatty acid profiles (Komagata & Suzuki, 1987), pre-dominant menaquinones (Collins, 1994), whole-organism sugars (Schaal, 1985) and muramic acid type (Uchida *et al.*, 1999).

Phylogeny. The almost-complete 16S rRNA gene nucleotide sequences of the six strains, as generated by Mincer *et al.* (2002), were aligned manually with corresponding sequences of representatives of all of the genera currently classified in the family *Micromonosporaceae* retrieved from the DDBJ/EMBL/GenBank databases, by using the PHYDIT program (available at <http://plaza.snu.ac.kr/~jchun/phydit/>). Evolutionary trees were inferred using the least-squares, maximum-likelihood, maximum-parsimony and neighbour-joining tree-making algorithms from the PHYLIP suite of programs (Felsenstein, 1993) and evolutionary distance matrices generated for the least-squares and neighbour-joining methods, as described by Jukes & Cantor (1969). The topologies of the resultant trees were evaluated by bootstrap analyses (Felsenstein, 1985) of the neighbour-joining dataset based on 1000 resamplings, using the SEQBOOT and CONSENSE options from the PHYLIP package (Felsenstein, 1993).

DNA base composition and DNA-DNA relatedness studies.

A standard procedure was used to extract genomic DNA from each of the test strains (Pitcher *et al.*, 1989). DNA from strains CNB-440^T and CNH-643^T was digested, dephosphorylated and analysed using a reversed-phase HPLC procedure (Tamaoka, 1994), as described by Kim *et al.* (1998), and molar G+C content was calculated after Mesbah *et al.* (1989). DNA-DNA relatedness studies were carried out between strain CNB-440^T and strains CNB-536 and CNH-898, between strain CNH-643^T and strains CNH-646 and CNH-964, and between strains CNB-440^T and CNH-643^T, using the identification service at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany), as described by Kim *et al.* (1999).

Phylogenetic analysis based on 16S rRNA gene spacer sequence comparisons.

PCR of intergenic spacer regions of the test strains was carried out as described by Zhang *et al.* (2001). The resultant PCR products were purified by using the Wizard PCR purification system (Promega), and then sequenced using DyeDeoxy Terminator Cycle sequencing kits (Applied Biosystems) and the complementary sequence of the universal primer 1525r described by Lane (1991). The resultant sequences were aligned manually against available corresponding sequences of members of the genera *Micromonospora* and *Verrucosipora* retrieved from the DDBJ/EMBL/GenBank databases. Evolutionary trees were inferred using the neighbour-joining tree-making algorithm from the PHYLIP suite of programs (Felsenstein, 1993) and an evolutionary distance matrix generated following Jukes & Cantor (1969).

RESULTS AND DISCUSSION

It was apparent from the present study that the six representative MAR 1 strains have properties consistent with their assignment to the family *Micromonosporaceae* (Goodfellow *et al.*, 1990; Koch *et al.*, 1996; Tamura *et al.*, 1997). To this end, all of the strains were aerobic, Gram-positive, non-acid-fast actinomycetes that formed a branched substrate mycelium and non-motile spores borne singly or in clusters, and contained *meso*-diaminopimelic acid and *N*-glycolated muramic acid in the wall peptidoglycan, arabinose, galactose and xylose as major sugars, diphosphatidylglycerol,

phosphatidylethanolamine, phosphatidylglycerol and phosphatidylinositol as diagnostic phospholipids, tetrahydrogenated menaquinones with nine isoprene units as the major isoprenologue, and complex mixtures of saturated, iso- and anteiso-fatty acids, but lacked mycolic acids.

The 16S rRNA gene sequence data confirmed and extended earlier results reported by Mincer *et al.* (2002) in showing that the MAR 1 strains formed a distinct monophyletic clade that corresponded to phyletic lines formed by representatives of the 12 genera with validly published names currently classified in the family *Micromonosporaceae* (Fig. 1). The

Fig. 1. Neighbour-joining tree (Saitou & Nei, 1987) based on nearly complete 16S rRNA gene sequences showing relationships between the MAR 1 strains and representatives of genera classified in the family *Micromonosporaceae*. Asterisks indicate branches of the tree that were also found using the least-squares (Fitch & Margoliash, 1967), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Kluge & Farris, 1969) methods. Numbers at nodes indicate the level of bootstrap support (%) based on a neighbour-joining analysis of 1000 resampled datasets; only values above 55% are shown. Bar, 0.02 substitutions per site.

status of the MAR 1 clade was supported by data from all of the tree-making algorithms and by a bootstrap value of 100 % in the neighbour-joining analysis. It was also evident that the representative MAR 1 strains had a phenotypic profile that separated them from members of all of the genera classified in the family *Micromonosporaceae* (Table 1). Indeed, the abundance, widespread distribution, phylogenetic divergence and physiological adaptations of the MAR 1 isolates suggest that they represent an ecologically significant component of the bacterial community in tropical marine sediments (Mincer *et al.*, 2002). The present data provide yet further evidence for recognizing that MAR 1 strains represent a new centre of taxonomic variation in the family *Micromonosporaceae* that merits generic status.

The 16S rRNA genes of the six representative MAR 1 isolates were relatively highly conserved as they shared similarities within the range 98·9–99·9 %, values which correspond to 2 and 17 nucleotide differences, respectively, at 1480 locations. Nevertheless, it is evident from Fig. 1 that the six strains can be assigned to two, albeit closely related, groups supported by high bootstrap values. In cases such as this, nucleotide sequences of alignable stretches of DNA that are less conserved than those of corresponding 16S rRNA gene sequences, such as sequence heterogeneity in 16S rRNA

gene spacer regions, can be used to highlight differences between strains (Gürtler & Stanisich, 1996; García-Martínez *et al.*, 2001; Zhang *et al.*, 2001). It is clear from Fig. 2 that the current 16S rRNA gene spacer sequence data provide a much better resolution of the two taxa delineated in the 16S rRNA gene study; the taxonomic integrity of the two sharply delineated groups was supported by high bootstrap values. However, the two groups merge to form a taxon that is distinct from corresponding groups containing representatives of the genera *Micromonospora* and *Verrucosipora*.

DNA–DNA relatedness studies provide a reliable way of distinguishing between representatives of actinomycete species that share high 16S rRNA gene similarities (Nam *et al.*, 2003; Jones *et al.*, 2004; Groth *et al.*, 2004). It is also well known that the minimum level of DNA–DNA relatedness required to circumscribe genomic species is taken to be 70 % (Wayne *et al.*, 1987). It was apparent from the DNA–DNA relatedness data that the members of the two phylogenetic subclades belong to distinct genomic species. Strains CNB-536 and CNH-898 shared similarity values of 99·8 and 88·8 % with reference DNA from strain CNB-440^T, whereas strains CNH-646 and CNH-964 had corresponding values of 84·4 and 86·2 % with strain CNH-643^T; strains CNB-440^T and CNH-643^T had a DNA–DNA relatedness

Table 1. Characteristics that differentiate the genus *Salinispora* from other genera classified in the family *Micromonosporaceae*

Genera: 1, *Salinispora*; 2, *Actinoplanes*; 3, *Asanoa*; 4, *Catellatospora*; 5, *Catenuloplanes*; 6, *Couchioplanes*; 7, *Dactylosporangium*; 8, *Longispora*; 9, *Micromonospora*; 10, *Pilimelia*; 11, *Spirilliplanes*; 12, *Verrucosipora*; 13, *Virgisporangium*. Data taken from Asano & Kawamoto (1986, 1988), Asano *et al.* (1989), Goodfellow (1989), Yokota *et al.* (1993), Tamura *et al.* (1994, 1995, 1997, 2001), Rheims *et al.* (1998), Kudo *et al.* (1999), Lee *et al.* (2000), Lee & Hah (2002) and Matsumoto *et al.* (2003). +, Present; –, absent; ND, not determined; Ara, arabinose; Gal, galactose; Man, mannose; Rha, rhamnose; Xyl, xylose. The abbreviations for menaquinones are exemplified by MK9(H₄), menaquinone with two of the nine isoprene units hydrogenated.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13
Require sea water for growth	+	–	–	–	–	–	–	–	–	–	–	–	–
Spore motility	–	+	–	–	+	+	+	–	–	+	+	–	+
Spore vesicles	–	+	–	–	–	–	+	–	–	+	–	–	+
Wall chemotype*	II	II	II	II	VI	VI	II	II	II	II	II	II	II
Major whole-organism sugars	Ara, Gal, Xyl	Ara, Xyl	Ara, Gal, Xyl	Ara, Gal and Xyl or only Xyl	Xyl	Ara, Gal, Xyl	Ara, Xyl	Ara, Gal, Xyl	Ara, Xyl	Ara, Xyl	Gal, Xyl	Man, Xyl	Ara, Gal, Man, Rha, Xyl
Fatty acid type†	3a	2d	2d	3b	2c	2c	3b	2d	3b	2d	2d	2b	2d
Major menaquinones (MK-)	9(H ₄)	9(H ₄), 10(H ₄)	10(H ₆), H ₈)	9(H ₄ , H ₆) or 10(H ₄)	10(H ₄), 11(H ₄)	9(H ₄)	9(H ₄ , H ₆ , H ₈)	10(H ₄ , H ₆)	9(H ₄ , H ₆), 10(H ₄ , H ₆)	9(H ₂ , H ₄)	10(H ₄)	9(H ₄)	10(H ₄ , H ₆ , H ₈)
Phospholipid type‡	II	II	II	II	III	II	II	II	II	II	II	II	II
G + C content (mol%)	70–73	72–73	71–72	70–72	70–73	70–72	72–73	70	71–73	ND	69	70	71

*According to the classification of Lechevalier & Lechevalier (1970).
†According to the classification of Kroppenstedt (1985).
‡According to the classification of Lechevalier *et al.* (1977).

Fig. 2. Neighbour-joining tree (Saitou & Nei, 1987) based on interspacer region sequences showing relationships between the MAR 1 strains and members of the genera *Micromonospora* and *Verrucospora*. Asterisks indicate branches of the tree that were also found using the least-squares (Fitch & Margoliash, 1967), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Kluge & Farris, 1969) methods. Numbers at nodes indicate the level of bootstrap support (%) based on a neighbour-joining analysis of 1000 resampled datasets; only values above 55% are shown. Bar, 0.1 substitutions per site.

value of 44.9%. Members of these genomic species could be distinguished by using a set of phenotypic properties. Strains CNH-643^T, CNH-646 and CNH-964, unlike the remaining strains, utilized L-proline, L-threonine, (+)-D-salicin and L-tyrosine as sole carbon sources and grew in the presence of rifampicin (25 µg ml⁻¹). Similarly, only strains CNB-440^T, CNB-536 and CNH-898 utilized (+)-D-galactose and inulin as sole carbon sources.

The genotypic and phenotypic data acquired in the present study, taken together with complementary data from Mincer *et al.* (2002), show that the six representative MAR 1 isolates form a new centre of taxonomic variation within the family *Micromonosporaceae* Krasil'nikov 1938 emend. Stackebrandt *et al.* 1997. The name proposed for this taxon is *Salinispora* gen. nov. Similarly, two species can be recognized in this genus, *Salinispora arenicola* sp. nov., the type species, and *Salinispora tropica* sp. nov.

Description of *Salinispora* gen. nov.

Salinispora (Sa.li.ni.spo'ra. N.L. adj. *salinus* saline; Gr. fem. n. *spora* a seed and, in bacteriology, a spore; N.L. fem. n. *Salinispora* a spore-forming bacterium originating from a saline habitat, indicating the marine habitat of the organism).

The description is based upon information taken from this study and from Mincer *et al.* (2002). Aerobic, Gram-positive, non-acid-fast actinomycetes that form extensively branched substrate hyphae (0.25–0.5 µm in diameter) that carry smooth-surfaced spores (0.8–3.8 µm in diameter) singly or in clusters (Fig. 3). All strains require sea water or a sodium-supplemented medium for growth. Colonies first

appear within 3–6 weeks depending on the growth medium. They are recognized by their lack of aerial hyphae and their pigmentation; the latter ranges from bright to pale orange to black on M1 medium. Spreading substrate mycelia are formed on low-nutrient media M4 and M5. Dark brown to black, bright orange or pink diffusible pigments are frequently produced. Colonies can become darkened during sporulation, with spores borne either sessily or on short sporophores. Vegetative hyphae are finely branched and do not fragment. Good growth occurs at 10–30 °C and pH 7–12. Arbutin (0.1%, w/v), casein (1%, w/v), elastin

Fig. 3. Scanning electron micrograph of *Salinispora tropica* CNB-440^T growing on ISP medium 2 supplemented with NaCl (3.5%, w/v), after incubation at 28 °C for 4 weeks. Magnification ×8000. Bar, 1 µm.

(3%, w/v), gelatin (0.4%, w/v) and starch (1%, w/v) are degraded, but not cellulose (1%, w/v), chitin (0.2%, w/v), tributyrin (0.1%, v/v) or xylan (0.4%, w/v). (+)-D-Cellobiose, α -lactose, (+)-D-melezitose and starch are used as sole carbon sources, but not (+)-D-fructose, (+)-D-mannose, (+)-D-ribose, (-)-L-sorbose or (+)-D-xylose (all at 1%, w/v). Good growth occurs in the presence of gentamicin sulphate (5 and 25 $\mu\text{g ml}^{-1}$), neomycin sulphate (5 $\mu\text{g ml}^{-1}$), streptomycin sulphate (5 $\mu\text{g ml}^{-1}$), novobiocin (5 and 25 $\mu\text{g ml}^{-1}$), penicillin G (5 $\mu\text{g ml}^{-1}$) and rifampicin (5 $\mu\text{g ml}^{-1}$). Strains contain *meso*-diaminopimelic acid as the diamino acid of the wall peptidoglycan; major amounts of arabinose, galactose and xylose in whole-organism hydrolysates; diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol and phosphatidylinositol as diagnostic polar lipids; tetrahydrogenated menaquinones with nine isoprene units as the major isoprenologue; and complex mixtures of saturated iso- and anteiso-fatty acids; but lack mycolic acids. The predominant fatty acids (as a percentage of total fatty acid content) are 13-methyltetradecanoic acid (iso- $\text{C}_{15:0}$; 3.7–13.3%), 14-methylpentadecanoic acid (iso- $\text{C}_{16:0}$; 36.4–53.5%), 16-methylheptadecanoic acid (iso- $\text{C}_{18:0}$; 3.5–10.2%), heptadecanoic acid ($\text{C}_{17:0}$; 4.1–7.8%) and 10-methyloctadecanoic acid ($\text{C}_{18:0}$ 10 methyl; 0.9–6.8%). The muramic acid moiety of the cell wall is glycolated. The G+C content of the DNA lies within the range 70–73 mol%. Unique nucleotide signatures are present at positions 207 (A), 366 (C), 467 (U) and 1456 (G) of the 16S rRNA gene. Abundant and widespread in tropical marine sediments. The type species is *Salinispora arenicola*. The genus is a member of the family *Micromonosporaceae* Krasil'nikov 1938 emend. Stackebrandt *et al.* 1997.

Description of *Salinispora arenicola* sp. nov.

Salinispora arenicola [ar.en.i.co'la. L. n. *arena* sand; L. suff. *-cola* (from L. n. *incola*) inhabitant, dweller; N.L. n. *arenicola* sand-dweller, indicating isolation from marine sediments].

The description is based on information taken from this study and from Mincer *et al.* (2002). Morphological, chemotaxonomic, genomic and general characteristics are as given in the genus description. The temperature range for growth is 10–30 °C, with an optimum at 20–28 °C. All strains require 25–50% sea water or a sodium-enriched medium for growth. Arbutin, L-proline, (+)-D-salicin, L-threonine and L-tyrosine are used as sole carbon sources for energy and growth, but not (+)-D-galactose or inulin. Grows in the presence of rifampicin (25 $\mu\text{g ml}^{-1}$). Isolated from coarse sand off the Bahamas. The type strain is CNH-643^T (= ATCC BAA-917^T = DSM 44819^T).

Description of *Salinispora tropica* sp. nov.

Salinispora tropica (tro'pi.ca. L. fem. adj. *tropica* tropical, pertaining to the tropics, the source of the isolates).

The description is based on information taken from this study and from Mincer *et al.* (2002). Morphological, chemotaxonomic, genomic and general characteristics are as given in the genus description. The temperature range for growth is 10–30 °C, with an optimum at 15–28 °C. (+)-D-Galactose and inulin are used as sole carbon sources for energy and growth. Does not grow in the presence of rifampicin (25 $\mu\text{g ml}^{-1}$). Isolated from coarse sand off the Bahamas. The type strain is CNB-440^T (= ATCC BAA-916^T = DSM 44818^T).

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