Jannaschia rubra sp. nov., a red-pigmented bacterium isolated from sea water

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A Gram-negative, slightly halophilic, strictly aerobic, chemo-organotrophic bacterium was isolated from Mediterranean sea water near Valencia (Spain). Comparison of the almost complete 16S rRNA gene sequence showed that strain 4SM3^T belonged to the *Roseobacter* group, with *Jannaschia helgolandensis* as its closest relative, with a similarity of 98.7 %. DNA–DNA hybridization analysis showed that the Mediterranean isolate had a level of relatedness of less than 42 % with *J. helgolandensis* and therefore that it represented a novel species of the genus *Jannaschia*. Phenotypic characteristics gave further evidence that the two organisms are not related at the species level. Isolate 4SM3^T grows on solid media as irregular pink–red colonies that penetrate into the agar. Cells are rods, motile by a tuft of polar flagella. The DNA base composition is 64.6 mol% G + C. Morphological, physiological and genotypic differences from related species support the description of a novel species, *Jannaschia rubra* sp. nov., with strain 4SM3^T (=CECT 5088^T=DSM 16279^T) as the type strain.

The genus *Jannaschia* was described by Wagner-Döbler *et al.* (2003) to accommodate two strains isolated from sea water sampled in the North Sea, with *Jannaschia helgolandensis* as the type and sole species. The genus was defined based on its phenotypic characteristics, including unique chemo-taxonomic features, and its isolated position on the phylogenetic branch of *Roseobacter* and related genera in the α -Proteobacteria. A second species, *Jannaschia cystaugens*, has been proposed by Adachi *et al.* (2004) that includes marine isolates from the Seto inland sea in Japan. A third species of the genus is proposed here to accommodate a strain obtained from Mediterranean sea water sampled on the Spanish east coast, near Valencia.

Strain 4SM3^T was isolated after direct plating of sea water dilutions on marine agar (MA; Scharlab) and incubation at

25 °C for 10 days. The sampling area was 3.2–4.8 km off the coast. Samples were taken by a diver with sterile bottles at 15–20 m water depth and transported under refrigeration to the laboratory within 3–4 h. The sample from which strain 4SM3^T was isolated was obtained in April 1990. The strain was phenotypically characterized and could not be ascribed to any recognized taxon in a previous numerical taxonomic study (phenon 45 in Ortigosa *et al.*, 1994).

Since then, the strain has been maintained at –80 °C, with cells suspended in marine broth (MB; Scharlab) plus 10 % glycerol, and also by lyophilization. Working strains were routinely cultured on MA and MB at 20–25 °C. Most methods for biochemical characterization used here were performed as previously described by Ortigosa *et al.* (1994) and Macián *et al.* (2001), except for Tween 80 and DNase tests, which were performed in media prepared with half-strength artificial sea water (ASW: 400 mM NaCl, 100 mM MgSO₄·7H₂O, 20 mM KCl and 20 mM CaCl₂·H₂O) (Baumann & Baumann, 1981), and sulfite oxidation, which was tested according with González *et al.* (1999). Bacteria grown on MA for 2–3 days were used for scanning electron microscopy. A cell suspension was prepared with half-strength ASW and adsorbed onto Isopore membrane filters. Filters were dehydrated with a graded series of

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Abbreviation: PHB, poly- β -hydroxybutyrate.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Jannaschia rubra* 4SM3^T is AJ748747.

Transmission and scanning electron micrographs of cells of strain 4SM3^T are available as supplementary material in IJSEM Online.

ethanol (50, 80 and 100 % ethanol), critical-point dried with CO₂ (Autosamdri-814) and sputter-coated with a gold-palladium film to a thickness of about 10 nm (Bio-Rad sputter coater). Samples were examined in a Hitachi S-4100 field emission scanning electron microscope with 7–15 mm working distance and at an acceleration voltage of 10 kV. Pictures were stored digitally and processed using EMIP 3.0. In parallel, samples were examined in a JEOL JEM-1010 transmission electron microscope at 60 kV after negative staining with 2 % (w/v) phosphotungstic acid at appropriate pH.

Cells are rod-shaped and motile in young cultures as observed by optical microscopy. Up to five monopolar flagella could be seen by transmission electron microscopy (Supplementary Fig. C in IJSEM Online). Binary division was observed on scanning electron micrographs (Supplementary Fig. H in IJSEM Online). Rosette formation was not observed, but cells tended to form tight aggregates. Bright granules were never seen on wet mounts of cells of different ages and grown under different culture conditions, suggesting that strain 4SM3^T does not accumulate poly- β -hydroxybutyrate (PHB).

Colonies on MA are initially whitish but they progressively develop a dark-red pigmentation as the cultures age and become more dense. Young (less than 6 days old) colonies are smooth and shiny and have a regular border, but they become rough with increasing pigmentation and penetrate into the agar, being difficult to remove and to disperse into aqueous solutions.

The organism is unable to ferment sugars under anaerobic conditions as determined on anaerobic Hugh & Leifson O/F medium with half-strength ASW. It does not reduce nitrate to nitrite in nitrate broth and it is unable to grow in denitrification medium of Baumann & Baumann (1981). Strain 4SM3^T showed a quick and clear positive response to the test for the presence of cytochrome oxidase, whereas *J. helgolandensis* has been described to give a weakly positive reaction (Wagner-Döbler *et al.*, 2003). In our laboratory we have confirmed that its response is much slower and less intense than that of strain 4SM3^T.

The range of salinities that support growth of strain 4SM3^T was tested in MA [which contains approximately 2 % (w/v) NaCl and 1.4 % (w/v) other salts]. The upper limit was resolved by adding increasing amounts of NaCl up to 6, 7, 8, 9 and 10 % (w/v) total salinity. Growth and pigmentation were observed in media containing up to 8 % (w/v) total salinity, after 7 days of incubation. In MA with 9 % (w/v) total salts, growth was observed only after 12 days and no pigment was produced. Strain 4SM3^T was unable to grow at 10 % (w/v) salinity. Thus, our isolate could be regarded as slightly halophilic. *J. helgolandensis* DSM 14858^T was included in the experiments, and we found that this strain was able to grow at up to 7 % (w/v) but not at higher salinity.

In order to determine the minimal amount of salts required for growth, MA was diluted with distilled water to produce media containing 0.34, 0.68, 1.02, 1.36 and 1.7 % (w/v) total salts (dilution factors of 0.1, 0.2, 0.3, 0.4 and 0.5, respectively). The loss of nutrients and agar by dilution was compensated for by adding the appropriate amounts of peptone, yeast extract and agar. Growth diminished progressively with decreasing salinity down to 0.34 % (w/v) salts. Pigmentation was observed even with 1.02 % (w/v) salts, but was not present at lower concentrations. *J. helgolandensis* DSM 14858^T was able to grow with 0.68 but not with 0.34 % (w/v) salts.

Ionic requirements were determined in salt tolerance agar (STA) containing 1 % (w/v) tryptone, 0.3 % (w/v) yeast extract and 1.5 % (w/v) agar with different combinations of salts: (i) STA with 2 % (w/v) NaCl, (ii) STA with 2 % (w/v) NaCl and 0.2 % (w/v) CaCl₂·2H₂O, (iii) STA with 2 % (w/v) NaCl and 0.9 % (w/v) MgCl₂·6H₂O and (iv) STA with 2 % (w/v) NaCl, 0.9 % (w/v) MgCl₂·6H₂O, 0.2 % (w/v) CaCl₂·2H₂O and 0.06 % (w/v) KCl (all concentrations are equal to those found in MB for these four salts). Strain 4SM3^T, as well as *J. helgolandensis* DSM 14858^T, was unable to grow after 7 days of incubation at 25 °C in STA with just NaCl or with NaCl plus CaCl₂·2H₂O, indicating that Na⁺ and Ca²⁺ ions together do not meet the salt requirements for growth of these organisms. Good growth was observed for both strains with the combination of NaCl and MgCl₂·6H₂O, suggesting that Na⁺ and Mg²⁺ ions are required. However, under these conditions, pigmentation was delayed in the case of strain 4SM3^T.

Growth at different temperatures was tested in both liquid (MB) and solid (MA) media at 4, 15, 25, 37 and 40 °C. Nutritional screening was carried out on basal medium agar (BMA), which contained 50 mM Tris/HCl (pH 7.5), 19 mM NH₄Cl, 0.33 mM K₂HPO₄·3H₂O, 0.1 mM FeSO₄·7H₂O and 1.3 % (w/v) purified agar (Oxoid) on half-strength ASW (Baumann & Baumann, 1981). Carbohydrates were added at 2 g l⁻¹ and the remaining compounds were added at 1 g l⁻¹. BMA was supplemented with 0.1 g yeast extract l⁻¹ because the organism was originally reported to be unable to grow on minimal medium (Ortigosa *et al.*, 1994), and incubation was prolonged until 14 days. Positive control plates were prepared with 5 g yeast extract l⁻¹ and negative control plates consisted of BMA plus 0.1 g yeast extract l⁻¹. Growth was scored as negative when growth was equal to or less than that in the negative control plates. It was noted that, for some carbon sources that supported growth, pigmentation was absent or delayed. The results are summarized in Table 1 and in the species description below.

The cellular fatty acid composition, determined by GLC at the DSMZ using a method described by Kämpfer & Kroppenstedt (1996), included 18:1 ω 7c as the major fatty acid (79.4 %), a characteristic common to all genera in the *Roseobacter* group. The second most abundant fatty acid in strain 4SM3^T was 18:0 (8.13 %), followed by cyclo 19:0 ω 8c

Table 1. Phenotypic characteristics that differentiate *Jannaschia rubra* sp. nov. from *J. helgolandensis* and *J. cystaogens*

+, Positive; –, negative; (+), weakly positive; ND, not determined. Data are from this study unless indicated.

Characteristic	<i>J. rubra</i>	<i>J. helgolandensis</i>	<i>J. cystaogens</i> *
Oxidase	+	(+)†	+
Pigmentation	Red	None†	Brown
Motility	+	–†	+
Cell shape	Rods	Irregular rods†	Irregular rods
White inclusions	–	+†	+
DNase	+	–	ND
Salinity range for growth (%)	0.34–9	0.68–8	2–7
Utilization of:			
Rhamnose	–	+	–
Maltose	(+)	–	–
Cellobiose	–	+	–
meso-Inositol	(+)	–	–
Arginine	(+)	–	ND
Citrulline	+‡	–	ND
L-Histidine	(+)	–	ND
Sarcosine	+	–	ND
Putrescine	+	–	ND
Fatty acids:			
19:0 cyclo	+	+†	–
18:1 ω 9c	–	–†	+

*Data from Adachi *et al.* (2004).

†Data from Wagner-Döbler *et al.* (2003).

‡Pigment was absent or appeared after 7 days of incubation.

(5.73 %), a cellular fatty acid also present in the type species of the genus *Jannaschia*. The latter fatty acid was not found by Adachi *et al.* (2004) in *J. cystaogens*. Other fatty acids found in strain 4SM3^T are 10:0 3-OH (2.32 %), 16:0 (0.68 %), 20:1 ω 7c (0.59 %) and three that are unknown: ECL 11.799 (2.19 %), ECL 15.273 (1.7 %) and ECL 17.606 (0.7 %).

Bacteriochlorophyll production was determined in acetone extracts using a Beckman DU-600 spectrophotometer as described by Takaichi *et al.* (1991). Extracts obtained from cells grown in the dark on MA plates and BMA plates containing D-sorbitol as the sole source of carbon and supplemented with 0.1 g yeast extract l^{–1} were analysed and bacteriochlorophyll *a* was not detected.

The DNA G+C content was determined by HPLC at DSMZ, following the procedure of Mesbah *et al.* (1989). Strain 4SM3^T has a G+C content of 64.6 mol%. This value is close to those determined for *J. helgolandensis* and *J. cystaogens*: 63.0 and 59.1 mol%, respectively (Wagner-Döbler *et al.*, 2003; Adachi *et al.*, 2004).

To investigate the genealogy of our isolate, comparative 16S

rRNA gene sequence analysis was performed. Isolation of genomic DNA, amplification of almost full-length 16S rRNA gene fragments and sequencing of the rRNA gene using a LICOR automated sequencer (MWG Biotech) were performed as described by Macián *et al.* (2001). Sequences were added to the 16S rRNA gene sequence databases of the Technical University Munich using the program package ARB (Ludwig *et al.*, 2004). Automated sequence alignments were inspected by eye and corrected manually using the sequence editor ARB_EDIT. Phylogenetic analyses using different tree-construction methods (maximum-parsimony, maximum-likelihood and distance matrix) and data subsets were performed using the appropriate ARB tools to test the robustness of local topologies (Ludwig *et al.*, 1998). As is commonly observed in such tree comparisons, variations in topology were detected, but none of these occurred among the closest relatives of our isolate. Fig. 1 shows the tree derived by distance matrix analysis, using the Jukes–Cantor correction.

Comparative analyses of the 16S rRNA gene sequence obtained in this study (deposited at the GenBank/EBI database under accession number AJ748747) confirmed the affiliation of strain 4SM3^T to the α -Proteobacteria, and more precisely to the genus *Jannaschia*. The closest 16S rRNA gene sequences were AJ438157, AJ534224 and AJ534225 (Fig. 1), all of which correspond to strains of *J. helgolandensis* (Wagner-Döbler *et al.*, 2003), bearing 98.7 % sequence similarity with strain 4SM3^T. Sequences from other species within the α -Proteobacteria, including *J. cystaogens*, showed similarity levels below 95.7 %.

DNA–DNA hybridizations were performed between strain 4SM3^T and *J. helgolandensis* DSM 14858^T. Experiments were performed using microplates as described by Ziemke *et al.* (1998). Colour development was measured at 405 nm using a Wallac Victor V2 plate reader (Perkin-Elmer). Hybridizations were performed at 60 °C. DNA–DNA relatedness between strain 4SM3^T and *J. helgolandensis* DSM 14858^T was 42 %, a mean value based on two independent hybridization experiments, confirming that the two strains are separated at the species level (Stackebrandt & Goebel, 1994).

Taking into consideration the distinct phenotypic and genetic properties summarized in Table 1, we conclude that the isolate represents a novel species of the genus *Jannaschia*. Phenotypic differences that support the consideration of strain 4SM3^T as representing a novel species include its characteristic pigment, its motility and development of flagella, its inability to produce PHB and the salinity range supporting growth (Table 1).

Description of *Jannaschia rubra* sp. nov.

Jannaschia rubra (ru'bra. L. fem. adj. *rubra* red).

Gram-negative, rod-shaped cells, about 1.0–2.0 $\times$ 0.5 μ m. Divides by binary division. Motile by means of three to five monopolar flagella. Growth on MA after 7 days of

Fig. 1. Phylogenetic relationship based on a distance matrix (Jukes–Cantor correction) of the 16S rRNA gene sequences of *Jannaschia rubra* sp. nov. and other α -Proteobacteria. All sequences are derived from type strains and accession numbers are shown in parentheses. The outgroup (more than 200 bacterial sequences) has been deleted to simplify the figure. Bootstrap values above 50% are shown at the left of nodes (percentages based on 1000 resamplings). Bar, 5% sequence divergence.

incubation leads to irregular, red colonies adhering to the agar. It does not swarm or luminesce. Requires at least 0–34% (w/v) marine salts and tolerates up to 9% (w/v) salts. Sodium and magnesium are required. Temperature range for growth is 4–25 °C. No growth detected at 37 °C or above. Oxidase-positive. Strict aerobic chemo-organotrophic bacterium. Does not reduce nitrate to nitrite or to N₂. Does not hydrolyse casein, gelatin, starch, Tween 80, alginate or lecithin, but shows DNase activity on DNase agar supplemented with half-strength ASW. Negative for arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase activities. Negative for H₂S production from thiosulfate, indole production from tryptophan and sulfite oxidation. Able to grow on BMA with the following compounds as carbon and energy sources: D-xylose, D-glucose, D-fructose, D-galactose, D-mannose, glycerol, D-mannitol, D-sorbitol, pyruvate, succinate, fumarate, malate, lactate, acetate, DL- β -hydroxybutyrate, L-leucine, L-serine, L-glutamate, γ -aminobutyric acid, L-ornithine, citrulline, L-aspartate, sarcosine and putrescine, provided that the medium is supplemented with a small amount of yeast extract (an indication that undetermined growth factors are required). Under the same conditions, the following carbon sources are weakly positive: maltose, D-gluconate, meso-inositol, glycerate, citrate, *cis*-aconitate, α -ketoglutarate, L-arginine and L-histidine. Pigmentation is absent or delayed when using pyruvate, L-glutamate, γ -aminobutyric acid, L-ornithine, citrulline, L-aspartate and glycerate. The following substrates are not used: D-ribose, L-arabinose, D-trehalose, L-rhamnose, cellobiose, sucrose, lactose, D-melibiose, amygdalin, D-glucuronate, D-galacturonate, N-acetylglucosamine, saccharate, propionate, *p*-hydroxybenzoate, glycine, L-threonine, L-tyrosine and L-alanine. The G+C content of the DNA of strain 4SM3^T is 64.6 mol%.

The type strain, 4SM3^T (=CECT 5088^T=DSM 16279^T), was isolated from Mediterranean sea water.

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