Full Paper

***Pseudomonas xanthomarina* sp. nov., a novel bacterium isolated from marine ascidian**

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Two *Pseudomonas*-like yellow-orange-pigmented non-fluorescent denitrifying strains KMM 235 and KMM 1447^T were isolated from marine ascidian specimens and investigated by a polyphasic approach to clarify their taxonomic status. On the basis of 16S rDNA gene sequence data the new isolates clustered with the *Pseudomonas stutzeri* species group with sequence similarities of >98%. The results of DNA-DNA hybridization and biochemical characterization showed genetic and phenotypic distinction between strains KMM 235 and KMM 1447^T and from the other validly described *Pseudomonas* species. Strain KMM 235 was found to be closely related to the type strain of *Pseudomonas stutzeri* in their phenotypic and genetic characteristics and represented, probably, a new *P. stutzeri* genomovar. It is proposed that strain KMM 1447^T be classified as a new species of the genus *Pseudomonas*, *Pseudomonas xanthomarina* sp. nov., with the type strain KMM 1447^T (=JCM 12468^T=NRIC 0617^T=CCUG 46543^T).

Key Words—*Pseudomonas*; *Pseudomonas stutzeri* cluster; *Pseudomonas xanthomarina* sp. nov.; sea ascidians

Introduction

The genus *Pseudomonas* has been reported to be a genetically and metabolically diverse bacterial group (Anzai et al., 2000; Kersters et al., 1996; Moore et al., 1996). *Pseudomonas* species of the rRNA-DNA similarity group I have been previously stated by Palleroni

(1984) to form the genus *Pseudomonas* (sensu stricto) within the γ subclass of the *Proteobacteria*. The formerly *Pseudomonas* species of the remaining four rRNA similarity groups (Palleroni, 1984) have been transferred into the other genera or formed the new taxa (Kersters et al., 1996). Among authentic *Pseudomonas* species *P. stutzeri*, formerly *P. perfectomarina* (Baumann et al., 1983; Rosselló-Mora et al., 1993), *P. alcaligenes*, *P. pseudoalcaligenes* (Palleroni, 1984), *P. alcaliphila* (Yumoto et al., 2001), and *P. balearica* (Bennasar et al., 1996) have been recovered from marine environments, mainly from seawater or sediment. *Pseudomonas* or *Pseudomonas*-like bac-

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teria are considered to be common members of microbial communities occupying marine plants or animals. At the same time the diversity of well-defined *Pseudomonas* species associated with marine invertebrates remains to be explored. During a polyphasic taxonomic study of bacteria isolated from sea environments we have identified deep-sea strains belonging to *P. stutzeri*, and *P. alcaligenes*, and proposed *Pseudomonas pachastrellae* sp. nov. to include strains associated with a marine sponge belonging to the genus *Pachastrella* (Romanenko et al., unpubl.).

In the present study we report the polyphasic characterization of two *Pseudomonas*-like strains KMM 235 and KMM 1447^T isolated from microbial associations with marine ascidian specimens. The bacteria KMM 235 and KMM 1447^T were found to be yellow-orange-pigmented, non-fluorescent, denitrifying and phylogenetically closely related to the *Pseudomonas stutzeri* cluster. Strain KMM 235 showed a high degree of genotypic and phenotypic similarity to *P. stutzeri* CIP 103022^T that did not allow a differentiation of strain KMM 235 from *P. stutzeri* CIP 103022^T for creating a new species. It is proposed that strain KMM 235 be assigned to the *P. stutzeri* cluster as a new genomovar. Strains KMM 235 and KMM 1447^T were distinct in their phenotypic characteristics and DNA relatedness from each other and strain KMM 1447^T was clearly separated from the other validly described *Pseudomonas* species. On the basis of phenotypic and molecular data obtained strain KMM 1447^T is described as a novel species, *Pseudomonas xanthomarina* sp. nov.

Materials and Methods

Two strains KMM 235 and KMM 1447^T were isolated from ascidian specimens, *Didemnum* sp. and *Halocynthia aurantium*, collected from the coastal waters near the Maldives in 1988, and from Troitsa Bay, Peter the Great Bay, the Sea of Japan, Russia, in 1992, respectively. Small amounts of invertebrate internal tissues were homogenated and aliquots of the diluted homogenates were spread on agar plates of seawater medium (SWM), containing (in g per liter), 5.0 peptone, 2.5 yeast extract, 1.0 glucose, 0.2 K₂HPO₄, 0.05 MgSO₄, 750 ml seawater, 250 ml distilled water, and 15.0 agar. The inoculated plates were incubated for 14 days at 28°C. Colonies were picked up, restreaked on the agar nutrient media and tested. The bacteria were

grown aerobically on Marine 2216 Agar (MA) or Marine Broth (MB; Difco), and Tryptic Soya Agar (TSA) at 25–28°C. They were also stored at –80°C in the liquid medium supplemented with 30% (v/v) glycerol. The strains designated KMM 235, and KMM 1447^T have been deposited in the Collection of Marine Microorganisms (KMM) of the Pacific Institute of Bioorganic Chemistry, Vladivostok, Russia. The following strains: *P. stutzeri* CIP 103022^T, *P. alcaligenes* CIP 101034^T, *P. pseudoalcaligenes* CIP 66.14^T, *P. mendocina* CIP 75.21^T, *P. chloritidismutans* DSM 13592^T, *P. balearica* DSM 6083^T, *P. stutzeri* DSM 50227, were obtained from the Collection de l'Institut Pasteur, Paris, France, and from the Deutsche Sammlung von Microorganismen und Zellkulturen, DSMZ, Braunschweig, Germany, and used in this study. Motility was observed by the hanging drop method. Cell morphology was examined by transmission electron microscopy from exponential phase cells grown in Tryptic Soya Broth (TSB). The cells were negatively stained with potassium phosphotungstate (1%, w/v; pH 7.0). The Gram-reaction, oxidase, catalase, hydrolysis of starch, casein, DNA, gelatin, and Tween 80 were tested according to the methods described by Smibert and Krieg (1994). Production of pyocyanin and fluorescent pigments were tested on King A and King B medium, respectively (King et al., 1954). Acid production from carbohydrates was examined by using the medium of Leifson (1963). API 20NE, API 32GN, and API ZYM (BioMérieux) were used to test additional biochemical properties and performed according to the manufacturer's instructions. The temperature range for growth was determined on TSA and MA at 4, 7, 12, 28, 30, 37, 41, 42, and 43°C. The pH range for growth (5.0–10.0) was tested using TSB. Sodium ion requirement and salt tolerance were examined on SWM prepared on the distilled water base supplemented with an appropriate amount of NaCl ranging from 0 to 15% (w/v). Antibiotic sensitivity was tested by a diffusion agar method on TSA plates using discs impregnated with the following antibiotics (content per disc): ampicillin 10 µg, benzylpenicillin 10 U, gentamicin 10 µg, kanamycin 30 µg, carbenicillin 25 µg, lincomycin 15 µg, oleandomycin 15 µg, polymyxin 300 U, streptomycin 30 µg, tetracycline 30 µg, and neomycin 15 µg. DNA was isolated by the procedure of Marmur (1961). DNA base composition was determined as described by Marmur and Doty (1962) and Owen et al. (1969). DNA-DNA relatedness was measured spectrophoto-

metrically by using the initial renaturation rate method (De Ley et al., 1970). Bacteria were grown on TSA at 28°C during 48 h to determine whole-cell fatty acids. Fatty acid methyl esters (FAME) were methylated by adding 1% NaOH in methanol, followed by heating 15 min at 60°C and then adding 5% HCl in methanol followed by heating for 15 min at 60°C. FAMES were extracted by 2× 0.2 ml hexane. Analyses of FAMES were performed using GC-9A gas chromatograph (Shimadzu, Japan) equipped with a capillary column (30 m × 0.25 mm I.D.) coated with Supelcowax-10 and SPB-5 phases (Supelco, USA). Identification of FAMES was accomplished by equivalent chain length values (Christie, 1988) and comparing the retention times of the samples to those of standards. 16S rDNA gene sequences of the strains tested were determined and compared with 16S rDNA sequences obtained from the EMBL/GenBank/DDBJ Databases as described by Shida et al. (1997). The DDBJ accession numbers for

the 16S rDNA sequences of strains KMM 1447^T and KMM 235 are AB176954 and AB176955, respectively.

Results and Discussion

Two marine isolates KMM 235 and KMM 1447^T were Gram-negative, yellow-orange-pigmented rod-shaped bacteria motile by means of a single polar flagellum. None of strains required sodium ions for growth; they grew in 0–6% (w/v) NaCl, but differed from each other by maximal NaCl concentrations supporting their growth. Unlike KMM 235, the strain KMM 1447^T was able to grow in 8% NaCl. It is worthy to note among the authentic *Pseudomonas* species *P. balearica* strains have been found to exhibit growth in up to 8.5% NaCl (Bennasar et al., 1996). The strain KMM 1447^T was psychrotolerant and characterized by growth at 4°C and absence of growth at 40 or 41°C. Both strains KMM 235 and KMM 1447^T reduced nitrate

Table 1. Phenotypic characteristics of strains KMM 1447^T, KMM 235, and related *Pseudomonas* species.

Feature	KMM 1447 ^T	KMM 235	<i>P. stutzeri</i>	<i>P. mendocina</i>	<i>P. alcaligenes</i>	<i>P. alkalicola</i>	<i>P. chloritidis-mutans</i>	<i>P. balearica</i>
Intracellular water-insoluble pigments	Yellow-orange	Yellow-orange	D	Yellow-orange	D	White	Pale brown	None
Arginine dihydrolase	–	–	–	+	+	+	ND	–
Denitrification	+	+	+	+	+	–	–	+
Growth in 8% NaCl	+	–	–	–	–	–	–	+
Growth at 4°C	+	–	–	–	–	+	+ ^a	– ^a
at 41°C	–	+	D	+	+	–	+ ^a	+ ^a
Hydrolysis of:								
Tween-80	–	–	+	–	–	+	+ ^a	+ ^a
Starch	–	+	+	–	–	–	+ ^a	+ ^a
Casein	–	–	–	–	–	+	– ^a	– ^a
Gelatin	–	–	–	–	D	+	– ^a	– ^a
Acid production from D-glucose	–	+	+	+	–	+	+ ^a	+ ^a
Utilization of:								
D-Glucose	+	+	+	+	–	+	+	ND
D-Mannitol	+	+	D	–	–	+	+	–
Maltose	+	+	+	–	–	–	+	+
D-Gluconate	+	–	D	+	–	ND	+	ND
L-Malate	+	+	+	–	+	+	–	+
L-Arginine	–	–	–	+	+	+	ND	ND
DNA G+C content, mol%	59.1	63.7	60.6–66.3	62.8–64.3	64–68	62.3–63.2	63.9	64.1–64.4

+, positive reaction; –, negative reaction; D, different reaction between strains; ND, no data available. Data from present study and from Palleroni (1984); Yumoto et al. (2001); Wolterink et al. (2002); and Bennasar et al. (1996).

^aData for the type strains of *P. chloritidis-mutans* DSM 13592^T and *P. balearica* DSM 6083^T were obtained from present study.

to nitrite and nitrite to nitrogen, but did not show hydrolytic reactions in respect to gelatin, starch, casein, DNA, Tween-80 or hemolysis. The other physiological and biochemical characteristics differentiating the novel marine isolates from each other and from related *Pseudomonas* species are summarized in Table 1 and Table 2.

The fatty acid composition of the new strains and *Pseudomonas stutzeri* CIP 103022^T was found to be similar and contained major fatty acids 16:0, 16:1 ω 9c, and 18:1. These results agree with data reported for *Pseudomonas* spp. (Oyaizu and Komagata, 1983; Uchino et al., 2001), *P. stutzeri* strains (Rainey et al., 1994), and *P. alcaliphila* (Yumoto et al., 2001). It should be noted, the strain KMM 1447^T contained 2.9% and 2.2% of 17:0cyc and 19:1cyc, respectively (Table 3). Bennasar et al. (1996) have reported the presence of the same cyclopropane fatty acids 17:0cyc and 19:1cyc (4.71% and 3.8%, respectively) for the *P. balearica* strains.

Comparative 16S rDNA gene sequence analysis showed affiliation of the new isolates to the genus *Pseudomonas* as it placed them in the *P. stutzeri* cluster (Fig. 1). Strain KMM 235 was closely related to *Pseudomonas stutzeri* genomovar 8 branch with 98.5% sequence similarity to *P. stutzeri* gv. 8 JM 300, whereas strain 1447^T clustered with the *Pseudomonas stutzeri* genomovar 3 branch, which includes the recently described species *P. chloritidismutans* DSM 13592^T (Wolterink et al., 2002). The strain KMM 1447^T shared 99.0% 16S rDNA sequence similarity to *P. chloritidismutans* DSM 13592^T and *P. stutzeri* gv. 3 DSM 50227. It should be noted, *P. chloritidismutans* DSM 13592^T and *P. stutzeri* gv. 3 DSM 50227 have been reported to belong to the same genomospecies because they share a high DNA-DNA similarity of 80.5%. However, *P. stutzeri* DSM 50227 was not assigned to the species *P. chloritidismutans* by Wolterink et al. (2002).

To confirm the taxonomic position of the new isolates DNA-DNA hybridization experiments between KMM 1447^T, KMM 235 and type strains of phylogenetically related *Pseudomonas* species were conducted. The levels of DNA relatedness between KMM 235 and related *Pseudomonas* species varied from 10 to 55% similarity, while strain KMM 1447^T exhibited lower (10–42%) DNA-DNA similarity values (Table 4). The values obtained being less than 70% accepted for the distinction of different species (Wayne et al., 1987)

Table 2. API ZYM activity of strains KMM 1447^T and KMM 235.

API ZYM test	KMM 235	KMM 1447 ^T
Alkaline phosphatase	—	+
Esterase C4	—	+
Esterase lipase C8	—	+
Trypsin	—	+
Phosphoamidase	—	+

All strains were positive for leucine arylamidase reaction; and negative for lipase C14, valine arylamidase, cystine arylamidase, α -chymotrypsin, acid phosphatase, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, *N*-acetyl- β -glucosaminidase, α -mannosidase, and α -fucosidase.

+, positive reaction; —, negative reaction.

Table 3. Cellular fatty acid profiles of strains KMM 1447^T, KMM 235, and *Pseudomonas stutzeri* CIP 103022^T.

Fatty acid	<i>P. stutzeri</i> CIP 103022 ^T	KMM 235	KMM 1447 ^T
12:0	6.8	7	7.8
14:0	1.0	0.7	1.4
16:0	22.0	19.1	21.8
16:1ω9c	24.5	27.5	30.4
17:0cyc	0.4	0.6	2.9
18:1	41.3	40.2	28.9
14:0-3OH	1.1	0.1	0.4
19:1cyc	0.7	0.8	2.2

The following fatty acids 12:1, 13:0i, 13:0, 13:1, 14:1 ω 7c, 15:0, 15:0i, 15:0a, 16:0i, 17:0i, 17:0a, 12:0-3OH, 17:0, 17:1 ω 8c, and 18:0 were detected at level <1%.

indicated the strains KMM 1447^T and KMM 235 do not belong to the same species and that they represent probably novel *Pseudomonas* species in the view of DNA-DNA hybridization results.

Phenotypic and chemotaxonomic characteristics of the strain KMM 235 were consistent with those of *P. stutzeri* CIP 103022^T (Tables 1, 3). Strain KMM 235 and *P. stutzeri* CIP 103022^T shared 98.5% 16S rDNA sequence similarity and a DNA relatedness value of 55% (Table 4). A high degree of genetic similarity between these two strains supported by their phenotypic homogeneity did not allow a discrimination of strain KMM 235 from *P. stutzeri* CIP 103022^T as a separate species. It was previously reported that strains of the

Fig. 1. Phylogenetic relationships of isolates KMM 1447^T, KMM 235, and some related *Pseudomonas* species on the basis of 16S rDNA sequences.

The branching pattern was generated by the neighbor-joining method. Numbers indicate bootstrap values greater than 700. Bar, 0.02 K_{nuc} unit.

Table 4. Levels of DNA relatedness among strains KMM 1447^T, KMM 235, and related *Pseudomonas* species.

Strain	G+C (mol%)	DNA-DNA similarity value with strains (%):	
		KMM 1447 ^T	KMM 235
KMM 1447 ^T	59.1	100	11
<i>P. stutzeri</i> CIP 103022 ^T	64.2	11	55
<i>P. stutzeri</i> DSM 50227	63.1	39	Nt
<i>P. chloritidismutans</i> DSM 13592 ^T	62.1	36	Nt
<i>P. balearica</i> DSM 6083 ^T	63.3	42	Nt
<i>P. alcaligenes</i> CIP 101034 ^T	65.4	15	33
<i>P. pseudoalcaligenes</i> CIP 66.14 ^T	65.7	10	21
<i>P. mendocina</i> CIP 75.21 ^T	64.6	11	17

Nt, not tested.

Pseudomonas stutzeri cluster form a phenotypically and genetically heterogeneous group composed of several genomovars (Bennasar et al., 1996; Ginard et al., 1997; Moore et al., 1996). It has been shown that *P. stutzeri* genomovar members are characterized by high DNA-DNA hybridization values between 40% and 60% while *P. stutzeri* CIP 103022^T displays a low level of similarity (below 20%) with the other *Pseudomonas* species (Rosselló et al., 1991; Rosselló-Mora et al.,

1996). Bennasar et al. (1996) have reported that 16S rDNA gene sequence similarities varied from 99.8% to 100% for the members of the same genomovar and from 98.0% to 99.7% for the strains belonging to different *P. stutzeri* genomovars. Taking into account the above-mentioned data we conclude that the strain KMM 235 most likely represents a new genomovar of *P. stutzeri*. It is evident further study should be performed to characterize strain KMM 235 as *P. stutzeri*

genomovar.

The strain KMM 1447^T shared a high 16S rDNA sequence similarity (99.0%) with *P. chloritidismutans* DSM 13592^T and *P. stutzeri* gv. 3 DSM 50227. However, low DNA-DNA reassociation values clearly demonstrated that strain KMM 1447^T could not be assigned to *P. chloritidismutans* or to the other phylogenetic relatives (Table 4). The genotypic discrimination from *P. chloritidismutans* DSM 13592^T was supported by a number of phenotypic differences, including the presence of growth in 8% NaCl, the inability to grow at 41°C, the ability to denitrify, and the inability to hydrolyze Tween-80 or starch, and the absence of acid production from D-glucose (Table 1). Denitrifying yellow-orange-pigmented bacterium KMM 1447^T shared phenotypic properties with the strains of *P. stutzeri*, *P. balearica*, *P. alcaliphila*, *P. alcaligenes*, and *P. mendocina*, but it could be differentiated in its minimal and maximal growth temperatures, growth in 8% NaCl (except for *P. balearica*, which is able to grow in 8% NaCl), enzyme activities and spectrum of substrates utilized, and lower DNA G+C content (Table 1).

On the basis of combined phenotypic and genotypic data a novel species, *Pseudomonas xanthomarina* sp. nov., is proposed to include strain KMM 1447^T.

Description of Pseudomonas xanthomarina sp. nov.

Pseudomonas xanthomarina (xan.tho.ma.ri'na. Gr. adj. *xanthus*, yellow; L. adj. *marina*, of the sea; *xanthomarina*, yellow-pigmented bacteria from the sea).

Gram-negative, aerobic, denitrifying, yellow-orange-pigmented, non-fluorescent, rod-shaped bacteria motile by means of a single polar flagellum. Cells are 1.6–1.8 µm long and 0.2–0.3 µm in diameter. Reactions for oxidase and catalase are positive. On nutrient agar media yellow-orange-pigmented smooth translucent colonies 3–5 mm in diameter are formed. Pyocyanin or fluorescent pigment production was not observed on King A or King B media, respectively. The strain grows in 0–8% NaCl at 4–37°C, does not grow in 10% NaCl or at 40–41°C. Acid is not formed from carbohydrates. Negative reactions for urease, lysine decarboxylase, ornithine decarboxylase, acetoin production, H₂S production, indole production, esculin and DNA hydrolysis, utilization of L-arabinose, D-mannose, trehalose, sucrose, phenylacetate, DL-norleucine, and N-acetylglucosamine, and positive for utilization of fructose, citrate, and DL-lactate. Other characteristics are shown in Table 1, Table 2, and Table 3. The strain

is susceptible to ampicillin 10 µg, gentamicin 10 µg, kanamycin 30 µg, carbenicillin 25 µg, lincomycin 15 µg, oleandomycin 15 µg, polymyxin 300 U, streptomycin 30 µg, tetracycline 30 µg, and neomycin 15 µg, and resistant to benzylpenicillin 10 U. The G+C content of the DNA is 59.1 mol% (determined by the thermal denaturation method). The type strain is KMM 1447^T (=JCM 12468^T=NRIC 0617^T=CCUG 46543^T). Isolated from the ascidian *Halocynthia aurantium* from coastal waters of Troitsa Bay, Peter the Great Bay, The Sea of Japan, Russia.

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