Taxonomic study of *Marinomonas* strains isolated from the seagrass *Posidonia oceanica*, with descriptions of *Marinomonas balearica* sp. nov. and *Marinomonas pollencensis* sp. nov.

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Novel aerobic, Gram-negative bacteria with DNA G + C contents below 50 mol% were isolated from the culturable microbiota associated with the Mediterranean seagrass *Posidonia oceanica*. 16S rRNA gene sequence analyses revealed that they belong to the genus *Marinomonas*. Strain IVIA-Po-186 is a strain of the species *Marinomonas mediterranea*, showing 99.77 % 16S rRNA gene sequence similarity with the type strain, MMB-1^T, and sharing all phenotypic characteristics studied. This is the first description of this species forming part of the microbiota of a marine plant. A second strain, designated IVIA-Po-101^T, was closely related to *M. mediterranea* based on phylogenetic studies. However, it differed in characteristics such as melanin synthesis and tyrosinase, laccase and antimicrobial activities. In addition, strain IVIA-Po-101^T was auxotrophic and unable to use acetate. IVIA-Po-101^T shared 97.86 % 16S rRNA gene sequence similarity with *M. mediterranea* MMB-1^T, but the level of DNA–DNA relatedness between the two strains was only 10.3 %. On the basis of these data, strain IVIA-Po-101^T is considered to represent a novel species of the genus *Marinomonas*, for which the name *Marinomonas balearica* sp. nov. is proposed. The type strain is IVIA-Po-101^T (=CECT 7378^T =NCIMB 14432^T). A third novel strain, IVIA-Po-185^T, was phylogenetically distant from all recognized *Marinomonas* species. It shared the highest 16S rRNA gene sequence similarity (97.4 %) with the type strain of *Marinomonas pontica*, but the level of DNA–DNA relatedness between the two strains was only 14.5 %. A differential chemotaxonomic marker of this strain in the genus *Marinomonas* is the presence of the fatty acid C_{17:0} cyclo. Strain IVIA-Po-185^T is thus considered to represent a second novel species of the genus, for which the name *Marinomonas pollencensis* sp. nov. is proposed. The type strain is IVIA-Po-185^T (=CECT 7375^T =NCIMB 14435^T). An emended description of the genus *Marinomonas* is given based on the description of these two novel species, as well as other *Marinomonas* species described after the original description of the genus.

The genus *Marinomonas* comprises Gram-negative bacterial strains distributed in different marine environments (Sanchez-Amat & Solano, 2005). Most recognized species of the genus have been isolated from seawater samples collected from different geographical locations: *Marinomonas communis* and *M. vaga* (formerly *Alteromonas communis* and *A. vaga*) from the Pacific

Ocean (Baumann *et al.*, 1972; van Landschoot & De Ley, 1983), *Marinomonas pontica* from the Black Sea (Ivanova *et al.*, 2005), *Marinomonas dokdonensis* from the East Sea of Korea (Yoon *et al.*, 2005) and *Marinomonas mediterranea* and *M. aquimarina* from the Mediterranean Sea (Solano *et al.*, 1997; Solano & Sanchez-Amat, 1999; Macián *et al.*, 2005). Some have been isolated from cold environments, such as subantarctic regions [*Marinomonas polaris* (Gupta *et al.*, 2006) and *M. ushuaiensis* (Prabakaran *et al.*, 2005)] or sea-ice samples [*Marinomonas primoryensis* (Romanenko *et al.*, 2003)]. The only species described as being associated

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains IVIA-Po-185^T, IVIA-Po-101^T and IVIA-Po-186 are EU188441, EU188448 and EU188440, respectively.

with higher organisms is *Marinomonas ostreistagni* (Lau *et al.*, 2006), in addition to some *M. aquimarina* strains (Macián *et al.*, 2005), isolated from oysters.

The marine plant *Posidonia oceanica* plays an important role in Mediterranean Sea ecosystems. *P. oceanica* meadows are in recession, although the causes of this process are not well understood (Marbà *et al.*, 2005; Duarte *et al.*, 2004). As part of a study to characterize the microbiota associated with *P. oceanica* (Marco-Noales *et al.*, 2006), samples of this plant were collected from different *P. oceanica* meadows along the coast of the Balearic Islands from March to November 2004. Samples were transported refrigerated to the laboratory under aseptic conditions. Associated microbiota were recovered by comminuting surface-disinfected leaves, roots or internal parts of rhizomes in small pieces in artificial seawater (ASW; Wolf & Oliver, 1992), according to the protocol of the EPPO (2004) for sampling plant material. Samples were plated on marine agar (MA; BD) and incubated at 25 °C for 24–72 h. By using this protocol, several hundred strains were isolated. The aim of the present study was to provide taxonomic characterization of the culturable microbiota associated with *P. oceanica* belonging to the genus *Marinomonas*.

In a first step, partial sequences of the 16S rRNA gene, corresponding approximately to the fragment between nucleotides 70 and 660 of the *Escherichia coli* gene, were obtained from all the isolated strains by the Molecular Diagnostics Center (MDC, Orihuela, Spain) as described by Martínez-Murcia *et al.* (1999). DNA sequences were determined by direct sequencing of the PCR products on an ABI 3100 Avant sequencer (Applied Biosystems).

Searches via the BLAST program (Altschul *et al.*, 1997) were performed with the sequences obtained, and those showing higher similarity to 16S rRNA gene sequences of *Marinomonas* species than to any other species were selected. In this way, approximately 15% of the strains that grew on MA were possible members of the genus *Marinomonas*. In this study, we report the characterization of strains IVIA-Po-101^T, IVIA-Po-185^T and IVIA-Po-186.

Genomic DNA was isolated from the selected strains and the almost-complete 16S rRNA gene was amplified and sequenced by the MDC. The sequences obtained have been deposited in the GenBank database. Phylogenetic analyses based on 16S rRNA gene sequences were conducted by using MEGA version 4 (Tamura *et al.*, 2007). The 16S rRNA gene sequences of the type strains of all recognized *Marinomonas* species, several related marine bacteria as well as *Vibrio cholerae* ATCC 14035^T as an outgroup were included in the analysis. All sequences were aligned by using the CLUSTAL W program within the MEGA software package, and distance matrices were generated by calculating the p-distance. Phylogenetic trees were constructed by using the neighbour-joining and maximum-parsimony methods. The reliability of the trees was evaluated by using a bootstrap analysis with 1000 resamplings.

DNA–DNA hybridization was performed by the Identification Services of the Deutsche Sammlung von Mikroorganismen und Zellkulturen. DNA was isolated by using a French pressure cell and was purified by chromatography as described by Cashion *et al.* (1977). DNA–DNA hybridization was carried out as described by De Ley *et al.* (1970) under consideration of the modifications described by Huß *et al.* (1983) by using a Cary 100 Bio UV/Vis spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with *in-situ* temperature probe. For determination of the G + C content, DNA was isolated, digested with nuclease P1 and dephosphorylated as described by Johnson (1994). The nucleosides obtained were analysed by HPLC (Kumura *et al.*, 1991).

For fatty acid analysis, bacterial isolates were cultivated on MA for 24 h at 25 °C prior to fatty acid extraction and methylation according to the Microbial Identification System procedure (MIDI Inc.). Extracted samples were analysed with a Hewlett Packard 5890 gas chromatograph. Fatty acid peaks were identified by the Microbial Identification System version 4, and profiles were compared with the database in the Sherlock System 3.10 (Microbial ID; MIDI Inc.). Unless otherwise indicated, biochemical, microscopic and physiological tests were performed as described by Baumann *et al.* (1972) and Solano & Sanchez-Amat (1999). Utilization of different compounds as carbon sources was assayed in BM mineral medium (Baumann *et al.*, 1972; Solano & Sanchez-Amat, 1999). In these assays, strain IVIA-Po-101^T was unable to grow in BM medium, suggesting that it could be auxotrophic for some growth factors. Medium MM101 was developed for cultivation of strain IVIA-Po-101^T and contained (l⁻¹) 40 g sea salts (Sigma), 1 g yeast extract and 0.07 g K₂HPO₄ (pH 7.5). For strain IVIA-Po-101^T, its capacity to use a compound as carbon source was determined by comparing growth in MM101 with and without the addition of that compound. Melanin synthesis was evaluated by checking brown to black pigmentation on MA, minimal medium, BM or MM101 depending on the strain, with glutamate as carbon and energy source plus 5 mM L-tyrosine as the substrate for melanogenesis (Solano *et al.*, 1997). Laccase activity was checked on 0.5% agarose plates containing 1 mM 2,6-dimethoxyphenol as described by Solano *et al.* (2000). Antimicrobial activity was screened by antibiogram tests against *E. coli* UM202 (Lucas-Elío *et al.*, 2006). Agar plugs from 2-week-old cultures on MA plates of the different *Marinomonas* strains were placed onto the *E. coli* lawn and the plates were incubated at 25 °C for 2 days. In all the tests performed, *M. mediterranea* MMB-1^T, from our own laboratory collection, and *M. pontica* 46-16^T, a generous gift of E. Ivanova, were used as controls and for comparison. These two strains were chosen because, as described below, they represent the *Marinomonas* species most closely related to strains IVIA-Po-101^T, IVIA-Po-185^T and IVIA-Po-186.

Table 1 shows differential characteristics between the strains studied. In some tests, all the strains studied gave the same results. For instance, the DNA G + C content was always below 50 mol%, as expected for the genus *Marinomonas*. All the strains required NaCl for growth and were able to use glucose, glutamate, α -ketoglutarate, fructose and malate as carbon sources. By contrast, none was able to use tyrosine, lactose, butyrate or valerate. The predominant cellular fatty acids (Table 2) were C_{18:1} ω 7c, C_{16:1} ω 7c and C_{16:0}, in accordance with previous results for recognized *Marinomonas* species (Ivanova *et al.*, 2000; Prabakaran *et al.*, 2005; Yoon *et al.*, 2005; Ivanova *et al.*, 2005). Another abundant fatty acid detected in all strains under our experimental conditions was C_{10:0} 3-OH (6–10 % of the total), indicating that it constitutes a characteristic shared by species in the genus *Marinomonas*. C_{14:0} and C_{18:0} were also detected in all strains studied, but at levels below 5 %.

Phylogenetic analysis indicated that strains IVIA-Po-101^T, IVIA-Po-185^T and IVIA-Po-186 clearly belonged to the genus *Marinomonas* (Fig. 1). The novel strains clustered with all previously described *Marinomonas* species in a branch clearly separated from other marine bacteria. This branch was identified with high reliability in the trees constructed by both the neighbour-joining and the maximum-parsimony methods. Within recognized *Marinomonas* species, IVIA-Po-185^T clearly constitutes a new branch. This strain shared highest 16S rRNA gene

sequence similarity (97.4 %) with *Marinomonas pontica* 46-16^T. However, DNA–DNA relatedness between the two strains was only 14.5 %. According to the recommendations of a threshold value of 70 % DNA–DNA relatedness for the definition of novel species (Wayne *et al.*, 1987), this result indicates that strain IVIA-Po-185^T represents a novel species of the genus *Marinomonas*. Table 1 shows phenotypic characteristics that can be used to differentiate this strain from recognized *Marinomonas* species. Characteristics of strain IVIA-Po-185^T that are uncommon in recognized *Marinomonas* species are the capacity to use *m*-hydroxybenzoate but not maltose as a carbon source. In addition, a unique chemotaxonomic marker of this strain compared with recognized *Marinomonas* species is the presence of the fatty acid C_{17:0} cyclo. Strain IVIA-Po-185^T is thus considered to represent a novel species of the genus *Marinomonas*, for which the name *Marinomonas pollencensis* sp. nov. is proposed.

Together with *M. mediterranea* MMB-1^T, strains IVIA-Po-186 and IVIA-Po-101^T formed a well-defined cluster in the genus *Marinomonas* (Fig. 1). Strains IVIA-Po-186 and *M. mediterranea* MMB-1^T shared 99.8 % 16S rRNA gene sequence similarity. No phenotypic differences were found between strain IVIA-Po-186 and *M. mediterranea* MMB-1^T for all the characteristics studied (Tables 1 and 2). *M. mediterranea* is unique in the genus *Marinomonas* because it shows melanin synthesis due to a tyrosinase (Solano *et al.*, 1997), laccase activity (Sanchez-Amat *et al.*, 2001) and

Table 1. Differential phenotypic characteristics between strains IVIA-Po-101^T, IVIA-Po-185^T and IVIA-Po-186 and the type strains of their closest relatives in the genus *Marinomonas*

Strains: 1, strain IVIA-Po-185^T; 2, *M. pontica* 46-16^T (unless indicated, data from Ivanova *et al.*, 2005); 3, *M. mediterranea* MMB-1^T; 4, strain IVIA-Po-186; 5, strain IVIA-Po-101^T. Data are from this study unless indicated. All strains required NaCl for growth and utilized glucose, glutamate, α -ketoglutarate, fructose and malate as carbon sources. None was able to utilize tyrosine, lactose, butyrate or valerate.

Characteristic	1	2	3	4	5
DNA G + C content (mol%; mean $\pm$ SD)	47.8 $\pm$ 0.4	46	46.3 $\pm$ 0.9	44.8 $\pm$ 0.8	43.4 $\pm$ 1.4
Cell shape	Helical	Helical*	Straight rods	Straight rods	Straight rods
Oxidase	–	+	–	–	–
NaCl tolerance (%)	0.5–10	0.5–10	1–5	1–5	1–5
Temperature range (°C)	5–37	4–33	15–25	15–25	15–25
Lipase	–	–	+	+	+
Protease (gelatinase)	–	–	+	+	+
Melanin synthesis	–	–	+	+	–
Laccase activity	–	–	+	+	–
Antimicrobial activity	–	–	+	+	–
Carbon source utilization					
Maltose	–	+	–	–	+
Galactose	+	–	–	–	+
Sucrose	–	+	+	+	+
Sorbitol	+	+	+	+	–
Glycine	–	+	–	–	–
<i>m</i> -Hydroxybenzoate	+	+	–	–	–
Acetate	+	+	+	+	–

*Data from the present study.

Table 2. Cellular fatty acid composition of strains IVIA-Po-101^T, IVIA-Po-185^T and IVIA-Po-186 and the type strains of related *Marinomonas* species

Taxa: 1, strain IVIA-Po-185^T; 2, *M. pontica* 46-16^T; 3, *M. mediterranea* MMB-1^T; 4, strain IVIA-Po-186; 5, strain IVIA-Po-101^T. Data are from this study. Values are percentages of total fatty acids. ND, Not detected.

Fatty acid	1	2	3	4	5
C _{10:0} 3-OH	6.96	6.54	9.29	9.81	9.55
Unknown 11.799	1.38	ND	5.59	6.01	5.36
C _{12:0}	9.82	ND	ND	ND	0.63
C _{12:1} 3-OH	ND	8.40	ND	ND	ND
C _{14:0}	1.22	1.60	3.14	3.85	4.21
C _{16:0}	16.53	11.73	16.20	15.02	16.61
C _{16:1} <i>ω</i> 7 <i>c</i>	14.56	34.26	22.50	22.12	24.25
C _{17:0} cyclo	3.45	ND	ND	ND	ND
C _{18:0}	3.72	2.27	1.72	2.63	1.48
C _{18:1} <i>ω</i> 7 <i>c</i>	38.42	22.90	36.48	39.75	37.21

an antimicrobial protein with lysine oxidase activity (Lucas-Elío *et al.*, 2006; Gomez *et al.*, 2006). Enzyme assays obtained in this study (data not shown) confirmed that strain IVIA-Po-186 also showed all these enzyme activities. Together, the above results indicate that strain IVIA-Po-186 is a member of *M. mediterranea*. This is the first report of the isolation of a strain of *M. mediterranea* from the microbiota of a plant. The type strain of this species, MMB-1^T, was isolated from seawater samples in a different geographical location (Solano *et al.*, 1997), although it was also an area in the Mediterranean Sea with *P. oceanica* meadows. Accordingly, it cannot be ruled out that strain MMB-1^T was also a component of the microbiota of this seagrass.

Apart from strain IVIA-Po-186, the strain most closely related phylogenetically to *M. mediterranea* is IVIA-Po-101^T (Fig. 1). *M. mediterranea* and strain IVIA-Po-101^T showed lipase and protease activities, characteristics not described for any other *Marinomonas* species. However, several relevant differential characteristics were found

between them. For example, strain IVIA-Po-101^T is the only auxotrophic *Marinomonas* strain reported so far. With regard to carbon source utilization, strain IVIA-Po-101^T differs from *M. mediterranea* in being able to use maltose and galactose, but not sorbitol or acetate (Table 1). More importantly, in contrast to the results for *M. mediterranea* strains, we did not detect melanin synthesis or tyrosinase, laccase or lysine oxidase activities in strain IVIA-Po-101^T. Strain IVIA-Po-101^T and *M. mediterranea* MMB-1^T shared 97.9 % 16S rRNA gene sequence similarity. However, the level of DNA–DNA relatedness between the two strains was only 12.6 %. According to the recommendations of a threshold value of 70 % DNA–DNA relatedness and the phenotypic differences observed, strain IVIA-Po-101^T is thus considered to represent a novel species of the genus *Marinomonas*, for which the name *Marinomonas balearica* sp. nov. is proposed.

This study has revealed that members of the genus *Marinomonas* form part of the microbiota of the marine plant *P. oceanica*. A strain of *M. mediterranea* has been detected, as well as two other strains representing novel species. Moreover, as a result of this study and the previous characterization of other *Marinomonas* species, an emended description of the genus is proposed.

Description of *Marinomonas balearica* sp. nov.

Marinomonas balearica (ba.le.a'ri.ca. L. fem. adj. *balearica* related to the Balearic Islands, from where the type strain was isolated).

Cells are Gram-negative rods that are motile by means of a single polar flagellum. Strictly aerobic, catalase-positive and oxidase-negative. Organic growth factors are required. Requires Na⁺ for growth; grows in the presence of 1–5 % NaCl. The temperature range for growth is 15–25 °C. No amylase or agarase activity. Lipase (Tween 80) and protease (gelatinase) activities are detected. Glucose, glutamate, α -ketoglutarate, fructose, maltose, galactose, sucrose and malate are used as carbon and energy sources, but not sorbitol, glycine, tyrosine, *m*-hydroxybenzoate, acetate, butyrate or valerate. The major fatty acids (>10 % of the

Fig. 1. Consensus neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic position of strains IVIA-Po-101^T, IVIA-Po-185^T and IVIA-Po-186 in relation to the type strains of representative *Marinomonas* species and other marine bacteria. Numbers at branches indicate bootstrap values (percentages) based on 1000 resamplings; only values >70% are shown. Asterisks indicate branches also identified by the maximum-parsimony method. Bar, 1 substitution per 100 nucleotide positions.

total) are C_{18:1}ω7c, C_{16:1}ω7c and C_{16:0}. The DNA G+C content of the type strain is 43.4 ± 1.4 mol%.

The type strain, IVIA-Po-101^T (=CECT 7378^T =NCIMB 14432^T), was isolated from the seagrass *Posidonia oceanica*.

Description of *Marinomonas pollencensis* sp. nov.

Marinomonas pollencensis (pol.len.cen'sis. N.L. fem. adj. *pollencensis* related to Pollença, the beach in the Balearic Islands from where the type strain was isolated).

Cells are curved, and motile by means of a single polar flagellum. Gram-negative, strictly aerobic, catalase-positive and oxidase-negative. Organic growth factors are not required. Requires Na⁺ for growth; grows in the presence of 0.5–10% NaCl. The temperature range for growth is 5–37 °C. No amylase, agarase, lipase (Tween 80) or protease (gelatinase) activities. Glucose, glutamate, α-ketoglutarate, galactose, fructose, sorbitol, *m*-hydroxybenzoate, malate and acetate are used as carbon and energy sources, but not maltose, sucrose, lactose, butyrate, valerate, glycine or tyrosine. The major fatty acids (>10% of the total) are C_{18:1}ω7c, C_{16:1}ω7c and C_{16:0}. The DNA G+C content of the type strain is 47.8 ± 0.4 mol%.

The type strain, IVIA-Po-185^T (=CECT 7375^T =NCIMB 14435^T), was isolated from the seagrass *Posidonia oceanica*.

Emended description of the genus *Marinomonas* Van Landschoot and De Ley 1984, 91^{VP} (effective publication Van Landschoot and De Ley 1983, 3071)

Since the description of the genus *Marinomonas* (Van Landschoot & De Ley, 1983), which took into consideration the phenotypic characteristics described by Baumann *et al.* (1972), novel strains and species have been isolated and described. This makes it necessary to emend the description of the genus to include the new data. For instance, for some characteristics, such as amylase, lipase and protease activities, for which the genus was described as negative, positive results have been detected in the novel species.

Members of the genus *Marinomonas* are Gram-negative, helical, curved or straight rods that are motile by means of polar flagella at one or both poles of the cells. Aerobic, having a strictly respiratory metabolism. Oxidase-positive or -negative. Na⁺ is required for growth. Do not accumulate poly-β-hydroxybutyrate. Glucose is used by all species and most of them use fructose, glutamate, acetate and malate. Do not utilize butyrate or valerate. Characteristic fatty acids are C_{18:1}ω7c, C_{16:1}ω7c, C_{16:0} and C_{10:0} 3-OH. The G+C content of the DNA is 41–50 mol%.

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