NOTE**Studies on the phylogenetic relationships of melanogenic marine bacteria: proposal of *Marinomonas mediterranea* sp. nov.**Francisco Solano¹ and Antonio Sanchez-Amat²Author for correspondence: Antonio Sanchez-Amat. Tel: +34 968 364955. Fax: +34 968 363963.
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The polyphenol oxidase (PPO) activities of the marine melanogenic strains MMB-1^T and 2-40 were compared. Both contained a multifunctional PPO able to oxidize a wide range of substrates. In spite of this similarity, phylogenetic studies based on 16S rRNA sequences showed that these strains are not closely related. Strain 2-40 is not related to any previously described genus. On the basis of these studies and morphological and physiological characteristics, it is proposed that strain MMB-1^T (= CECT 4803^T = ATCC 700492^T) represents a new species in the genus *Marinomonas*, *Marinomonas mediterranea* sp. nov.

Keywords: Melanin, polyphenol oxidases, strain MMB-1^T, strain 2-40, *Marinomonas mediterranea* sp. nov.

In recent years, interest has increased greatly in marine micro-organisms as valuable sources of biomaterials with biotechnological and pharmaceutical applications. These materials include extracellular polymers such as polysaccharides and melanin pigments (Weiner, 1997), although the range of biomolecules has yet not been fully explored. In the case of melanins, marine micro-organisms are interesting not only as a source of the final pigment but also because of the enzymes involved in melanin synthesis, namely polyphenol oxidases (PPO). These enzymes can oxidize a wide range of phenolics, amines and other aromatic compounds (Solano *et al.*, 1997) and therefore have important biotechnological applications related to the polymerization of phenols in liquids, oxidation of dyes, pulp bleaching and lignin and lignosulfonate treatments (Berka *et al.*, 1997).

Few melanogenic marine bacteria have been described. On the basis of the pathway of melanin formation employed, they can be classified into two major groups depending on the involvement of authentic PPO in the synthetic pathway. The first group is taxonomically well known and is characterized by the absence of PPO activity; *Shewanella colwelliana* and *Vibrio cholerae*

belong to this group. They synthesize melanins through the excretion of homogentisate, a *p*-dihydroxyphenolic intermediate of L-tyrosine catabolism that is easily oxidized in slightly basic media to yield a yellowish pigment called pyomelanin (Coon *et al.*, 1994; Kotob *et al.*, 1995; Ruzafa *et al.*, 1994, 1995; Sanchez-Amat *et al.*, 1998). Marine melanogenic bacteria expressing authentic PPO activity form the other group. These enzymes are able to catalyse the formation of dark-brown to black melanins with higher protective capacity than pyomelanins and therefore have potentially greater biotechnological usefulness.

So far only two marine strains of this second group have been described. Strain 2-40 was first isolated and described as a polysaccharide-digesting bacterium that produced a melanin-like pigment (Andrykovitch & Marx, 1988). A tyrosinase activity was identified in this micro-organism (Kelley *et al.*, 1990). In the case of the other strain, MMB-1^T, the existence of a PPO system formed by two different enzymes has recently been described (Solano *et al.*, 1997). One of the enzymes is a pluripotent PPO sharing characteristics of laccases (EC 1.10.3.2) and tyrosinases (EC 1.18.14.1) that is able to oxidize a wide range of compounds, whereas the second enzyme seems to be a classical tyrosinase activated by SDS (Sanchez-Amat & Solano, 1997). This feature is common to plant and amphibian tyrosinases (Moore & Flurkey, 1990; Wittenberg & Triplett, 1985).

The taxonomic positions of these melanogenic

Abbreviations: ABTS, 2,2'-Azinobis-(3-ethylbenzothiazoline-6-sulfonate); 2,6-DMP, 2,6-dimethoxyphenol; L-DOPA, 3,4-dihydroxy-L-phenylalanine; PHB, poly- β -hydroxybutyrate; PPO, polyphenol oxidase.

The GenBank accession numbers for the 16S rRNA sequences of strains MMB-1^T (complete) and 2-40 (first 519 nucleotides) are AF063027 and AF063028.

Table 1. Specific activities of extracts from strains 2-40 and MMB-1^T for the oxidation of monophenolic and diphenolic compounds

Strains were grown until stationary phase in 2216 marine broth (Difco). In the case of strain 2-40, 0.2% agar (Oxoid no. 3) was added. Enzyme activities were determined as previously described (Solano *et al.*, 1997) and 1 unit is defined as the amount of enzyme that catalysed the oxidation of 1 μmol substrate min^{-1} . Activities are given as $\text{mU mg protein}^{-1}$. ABTS, 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate).

Substrate	2-40	MMB-1 ^T
L-Tyrosine	3.4 $\pm$ 1.3	17 $\pm$ 6
L-Tyrosine + SDS	2.9 $\pm$ 1.5	83 $\pm$ 10
L-DOPA	39 $\pm$ 12	51 $\pm$ 24
L-DOPA + SDS	43 $\pm$ 17	232 $\pm$ 80
2,6-DMP	161.5 $\pm$ 27	105 $\pm$ 37
Syringaldazine	37.3 $\pm$ 8	91 $\pm$ 14
Guaiacol	41.2 $\pm$ 13	9 $\pm$ 3
ABTS	0.7 $\pm$ 0.4	1.3 $\pm$ 0.6

strains are uncertain. It had been suggested that strain 2-40 could be related to the genus *Alteromonas* (Andrykovitch & Marx, 1988). Most of the phenotypic taxonomic features of strain MMB-1^T have been reported previously (Solano *et al.*, 1997). These characteristics indicated that it might also be assigned to the genus *Alteromonas*, as described in *Bergey's Manual of Systematic Bacteriology* (Baumann *et al.*, 1984).

The presence of PPO activity in both strains and their taxonomic similarity to *Alteromonas* might suggest a relationship between these two observations. The major aim of this work was to clarify the taxonomic positions and phylogenetic relationships of strains 2-40 and MMB-1^T. To approach this problem, the PPO activities of both strains were compared and the 16S rRNA sequences were determined.

The capacity to oxidize different phenolic compounds was assayed in cellular extracts of strains 2-40 and MMB-1^T. In both cases, all substrates assayed were oxidized, including syringaldazine and L-tyrosine, which are considered specific to laccases and tyrosinases, respectively (Table 1). The main difference observed was that, unlike in extracts from strain MMB-1^T, the oxidation of L-tyrosine and 3,4-dihydroxy-L-phenylalanine (L-DOPA) in the extracts of strain 2-40 was not affected by the addition of SDS. In strain MMB-1^T this characteristic has been attributed to an SDS-activated tyrosinase (Solano *et al.*, 1997).

It has been reported that the oxidation of 2,6-dimethoxyphenol (2,6-DMP) and syringaldazine in strain MMB-1^T is due to a multifunctional PPO (Sanchez-Amat & Solano, 1997). In order to check this

possibility in strain 2-40, cellular extracts of this strain were subjected to SDS-PAGE under the same conditions described elsewhere for strain MMB-1^T (Solano *et al.*, 1997; Sanchez-Amat & Solano, 1997). Two well-defined bands of PPO activity were clearly evident (result not shown). Both bands were stained by all substrates assayed, L-tyrosine, L-DOPA, syringaldazine and 2,6-DMP. This clearly indicated that strain 2-40 also expresses a multifunctional PPO sharing properties of tyrosinases and laccases. In contrast to MMB-1^T, the molecular masses of those two bands (55 and 110 kDa) strongly suggested the existence of an association/dissociation equilibrium of a single enzymic species resulting from the presence of SDS in the electrophoresis.

The presence of such a multifunctional PPO, able to oxidize a wide range of aromatic compounds including substrates specific for laccases and tyrosinases, has not previously been described in any organism other than the bacterial strain MMB-1^T. Laccase activity has been described in fungi and higher plants and in just one non-marine bacterial strain (Givaudan *et al.*, 1993; Thurston, 1994). Tyrosinases are even more widely distributed in many different organisms (Robb, 1994). The presence of this bacterial-specific multifunctional PPO in strains 2-40 and MMB-1^T prompted to us to analyse the phylogenetic relationship between these bacterial strains to explore whether the expression of this kind of enzyme might be restricted to a specific group of micro-organisms.

Genomic DNA was extracted from strains MMB-1^T and 2-40 and the corresponding 16S rDNA was amplified and sequenced by MIDI Labs (Newark, USA). A complete sequence consisting of 1534 nucleotides was obtained for strain MMB-1^T, whereas for strain 2-40 it was only possible to sequence the first 519 nucleotides. These sequences were compared with 16S rRNA or rDNA sequences in public databases by using the BLASTN 1.4.11 program (Altschul *et al.*, 1990), as well as with sequences contained in the Ribosomal Database Project library (Maidak *et al.*, 1997). In all cases, the sequences showing greatest identity to these new sequences were from micro-organisms belonging to the γ -subclass of the *Proteobacteria*.

The phylogenetic positions of strains MMB-1^T and 2-40 were studied in detail by including in the analysis the strains that gave the closest matches through the searches mentioned above and also some other species representing important groups of marine bacteria. The sequences were aligned by using the CLUSTAL W 1.7 program available at the Baylor College of Medicine Search Launcher (Thompson *et al.*, 1994; Smith *et al.*, 1996). Finally, genetic analysis was performed by using the package MEGA (Kumar *et al.*, 1993).

In the case of strain MMB-1^T, the highest level of identity was observed to the two species of the genus *Marinomonas*, *Marinomonas vaga* (95.7 $\pm$ 0.5%) and

Table 2. Percentage identities between rDNA or rRNA sequences of strain MMB-1^T and other marine bacteria

Identities were calculated from full-length 16S rDNA or rRNA sequences (approx. 1450 nt). Sequences compared are: 1, strain MMB-1^T (GenBank accession no. AF063027); 2, *Marinomonas vaga* (X67025); 3, *Marinomonas communis*; 4, *Marinobacterium georgiense* (U58339); 5, *Oceanospirillum linum* (M22365); 6, *Oceanospirillum beijerinckii*; 7, *Microbulbifer hydrolyticus* (U58338); 8, strain SCB11 (Z31658); 9, *Marinobacter hydrocarbonoclasticus* (X67022); 10, *Vibrio cholerae* (X74695); 11, *Pseudoalteromonas haloplanktis* (X67024); and 12, *Alteromonas macleodii* (X82145). Sequences 3 and 6 were determined by C. R. Woese (unpublished results) and are available at RDP (Maidak *et al.*, 1997).

	2	3	4	5	6	7	8	9	10	11	12
1	95.7±0.5	95.6±0.5	90.6±0.8	91.5±0.7	90.4±0.8	89.2±0.8	88.8±0.8	89.4±0.8	81.7±1.0	88.2±0.8	85.8±0.9
2		97.3±0.4	90.5±0.8	90.4±0.8	89.1±0.8	88.9±0.9	88.4±0.9	89.1±0.8	81.4±1.0	87.4±0.9	85.6±0.9
3			91.2±0.8	91.4±0.7	90.5±0.8	89.8±0.8	89.0±0.8	89.5±0.8	82.3±1.0	88.4±0.9	86.6±0.9
4				89.8±0.8	89.8±0.8	88.9±0.8	89.7±0.8	89.2±0.8	81.0±1.1	88.0±0.9	85.7±1.0
5					97.4±0.4	90.6±0.8	89.9±0.8	90.7±0.8	82.3±1.0	87.8±0.9	85.5±1.0
6						90.9±0.8	89.6±0.8	90.9±0.8	82.9±1.0	87.1±0.9	85.0±1.0
7							90.8±0.8	91.6±0.8	81.5±1.1	86.8±0.9	85.5±1.0
8								88.7±0.9	82.0±1.0	87.9±0.9	86.1±0.9
9									80.7±1.1	88.0±0.9	85.5±1.0
10										82.6±1.0	82.1±1.0
11											89.5±0.8

Fig. 1. Phylogenetic position of strain MMB-1^T in the context of other marine bacteria. The tree was generated by the neighbour-joining method from whole 16S rRNA sequences. Numbers indicate the support of branches by bootstrap analysis. Asterisks indicate branches also identified by the maximum parsimony method. Only values greater than 90% are included.

Marinomonas communis (95.6±0.5%) (Table 2). In both cases, the identity was slightly lower than that between *M. vaga* and *M. communis* (97.3±0.4%). Apart from the two *Marinomonas* species, other bacteria closely associated with strain MMB-1^T were members of the genus *Oceanospirillum* and *Marinobacterium georgiense* (González *et al.*, 1997), which showed identities slightly greater than 90%. Further genetic analysis was performed by using different protocols available in the MEGA program for creating phylogenetic trees. In all cases, it was observed that

strain MMB-1^T formed a well-defined phylogenetic unit with the two *Marinomonas* species described so far (Fig. 1).

Since the introduction of the genus *Alteromonas* by Baumann *et al.* (1972), this genus has been subjected to several rearrangements to accommodate the high level of heterogeneity detected as data on new potential members are accumulated. In this respect, the genus *Marinomonas* was created on the basis of rRNA–DNA hybridization experiments in response to the low level of similarity observed between two former species of the genus *Alteromonas*, *Alteromonas vaga* and *Alteromonas communis*, and other species (Van Landschoot & De Ley, 1983). Further phylogenetic analysis using 16S rRNA sequences confirmed the *Alteromonas*/*Marinomonas* split and allowed the further division of the remaining species into the genus *Alteromonas*, with the single species *Alteromonas macleodii*, and a new genus *Pseudoalteromonas*, the type species of which is *Pseudoalteromonas haloplanktis* (Gauthier *et al.*, 1995).

Our phylogenetic analysis clearly supports the separation of the genus *Marinomonas* from the strains previously included in the genus *Alteromonas* (Fig. 1). It also shows that strain MMB-1^T is closely related to the *Marinomonas* species already described. It has been proposed that rRNA identities of 97% or more may correspond to DNA reassociation values greater than 70%, thus indicating that the micro-organisms showing this level of identity could be considered to be different strains of the same species (Stackebrandt & Goebel, 1994). Since the identities of strain MMB-1^T to the two *Marinomonas* species were lower than this value, if this criterion is assumed then this strain constitutes a third species in the genus.

From the classical taxonomic approach, there are some important differences between strain MMB-1^T

Table 3. Taxonomic characteristics of strain MMB-1^T compared with other *Marinomonas* species

Data were taken from Solano *et al.* (1997) (MMB-1^T) and Baumann *et al.* (1972) (*M. vaga* and *M. communis*). PHB, poly- β -hydroxybutyrate.

Characteristic	MMB-1 ^T	<i>M. vaga</i>	<i>M. communis</i>
Cell shape	Straight rod	Straight rod	Curved rod
Polar flagellation	+	+	+
Anaerobic growth	—	—	—
Na ⁺ requirement	+	+	+
PHB accumulation	—	—	—
Denitrification	—	—	—
NO ₃ ⁻ reduction to NO ₂ ⁻	+	—	—
Luminescence	—	—	—
Pigmentation	+	—	—
Activity of:			
Cytochrome <i>c</i> oxidase	—	—	+
Lipase	+	—	—
Gelatinase	+	—	—
Amylase	—	—	—
Utilization of:			
D-Glucose	+	+	+
D-Sorbitol	+	+	+
<i>m</i> -Hydroxybenzoate	—	+	+
Succinate	+	+	+
Malate	+	+	+
DNA G+C content (mol %)	46.3 ± 0.9	45.4–47.6	44–47

Fig. 2. Phylogenetic position of strain 2-40 in the context of other marine bacteria. The tree was generated by the neighbour-joining method from the sequence of the first 500 nucleotides of the 16S rRNA. Numbers indicate the support of branches by bootstrap analysis. Only values greater than 95% are included.

and the two other *Marinomonas* species (Table 3). The four most significant differences are that strain MMB-1^T is melanogenic, reduces nitrate, is unable to metabolize *m*-hydroxybenzoate and expresses gela-

tinase and lipase activities. According to these observations, we propose that strain MMB-1^T (= CECT 4803^T = ATCC 700492^T) should be assigned to a new species, *Marinomonas mediterranea* sp. nov., as the type strain.

The taxonomic status of strain 2-40, the other melanogenic strain, was studied by using only the first 519 nucleotides of the 16S rDNA sequence. The percentage identity to other sequences was first calculated. It is worth mentioning that this portion of the sequence seems to be sufficient to get a good estimate of the overall identity. For example, the identity between strain MMB-1^T and *M. vaga* was 94.5 ± 1.2% in the first 500 nucleotides and 95.7 ± 0.5% for the whole sequence. Comparison of the 16S rRNA sequence from strain 2-40 with available sequences always gave the closest match to the sequence of strain SCB11 (accession no. Z31658; A. Rehnstam-Holm, S. Backman, F. Azam & A. Hagstrom, unpublished results). However, the sequence identity, even in this case, was as low as 92.2 ± 1.3%. The identities between the 16S rRNA sequences of strain 2-40 and the *Marinomonas* species or strain MMB-1^T were even lower, in the range 89–91%. Compared with *A. macleodii* and *P. haloplanktis*, the identities obtained were 84.9 ± 1.8 and 88.9 ± 1.6%, respectively.

The phylogenetic tree generated by the neighbour-joining method clearly indicates that strain 2-40 and strain SCB11 are closely related (Fig. 2). The low

sequence identity between these two strains and other micro-organisms indicates that strain 2-40 may represent a new, previously undescribed genus.

In spite of the taxonomic similarities of this strain to the genus *Alteromonas* first described by Andrykovitch & Marx (1988), some characteristics of this strain were quite different to those of other strains belonging to the genus *Alteromonas*. In this respect, 2-40 did not grow in liquid marine medium 2216 (Difco) unless 0.2% agar or starch was added. This is rather remarkable, since it did not grow even when 0.2% glucose was added to the same marine culture medium. Furthermore, the addition of this amount of D-glucose had an inhibitory effect on growth in media with agar or starch. Growth inhibition in the presence of monosaccharides suggests that strain 2-40 may be an example of a marine bacterium adapted to oligotrophic conditions and may help to explain why other similar strains have not been described. These data support the divergence suggested by the 16S rRNA data between 2-40 and the genus *Alteromonas*.

In summary, the present work shows that strain 2-40 shares with strain MMB-1^T the property of having a multifunctional PPO. In spite of this feature and some other phenotypic similarities, the 16S rRNA-based genetic distance between the strains was very high, suggesting that this type of enzyme is not restricted to a defined taxonomic unit. MMB-1^T seems to belong to the genus *Marinomonas* and we propose the name *M. mediterranea* for this strain. However, strain 2-40 seems to belong to a new genus not yet described. Our results also suggest that different aerobic marine bacteria could be a valuable source of multifunctional PPO, a type of enzyme so far found only in this type of micro-organism. This PPO has a wider range of substrate specificity than fungal laccases or tyrosinases and so could potentially be very useful in different biotechnological applications. Whether this kind of PPO is also present outside the eubacterial kingdom remains to be elucidated.

Description of *Marinomonas mediterranea* sp. nov.

Marinomonas mediterranea (med.i.terr.an'e.a. L. gen. n. *mediterranea* of the Mediterranean, as the type strain was isolated from the Mediterranean Sea in the region of Murcia, on the south-eastern Spanish coast).

Most of the taxonomic characteristics of this strain are summarized in Table 3. Cells are Gram-negative rods, motile by a single polar flagellum. Does not grow under anaerobic conditions. Cytochrome oxidase-negative. Unable to denitrify. Shows lipase and weak gelatinase activities. Able to metabolize D-glucose, D-mannose, D-sorbitol, citrate, β -hydroxybutyrate, succinate, glycerol and malate. The DNA G+C content of the type strain is 46.3 ± 0.9 mol%, as determined by total hydrolysis and HPLC. Produces melanins and contains a pluripotent polyphenol oxidase and a tyrosinase activated by SDS. The GenBank accession

number for the 16S rRNA sequence of strain MMB-1^T is AF063027. The type strain of *Marinomonas mediterranea* is MMB-1^T (=CECT 4803^T =ATCC 700492^T).

Acknowledgements

This work has been supported by the grant PB97-1060 from the CICYT, Spain.

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