Phylogenetic characterization of marine bacterium strain 2-40, a degrader of complex polysaccharides

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The marine bacterium strain 2-40 was isolated from the salt marsh cord grass, Spartina alterniflora, in the Chesapeake Bay watershed, VA, USA. It is Gram-negative, requires sea salts and is a strict aerobe. It degrades numerous complex polysaccharides and synthesizes eumelanin. By 16S rDNA analysis, the isolate was shown to be a member of the γ-subclass of the Proteobacteria, related to Microbulbifer hydrolyticus and to a cellulolytic nitrogen-fixing bacterium.

Keywords: phylogeny, tyrosinase, 16S rDNA, complex polysaccharides, hydrolytic enzymes

In the salt marsh ecosystem, complex polysaccharides (CP) derived from plants are degraded by microorganisms, principally bacteria, once the plant detritus reaches the aquatic environment (Benner et al., 1986). Due to the high primary productivity and abundance of vascular-plant-derived lignocellulosic material, the degradation of these polymers is an important environmental process relating to the food web and carbon cycling. To learn more about the type of organisms involved in the degradation of CP, bacteria were isolated from decaying Spartina alterniflora (Andrykovitch & Marx, 1988).

One isolate stood out for its ability to quickly degrade agar and other CP: strain 2-40 (ATCC 43961) was isolated on 1% peptone-half-strength-seawater agar and other CP: strain 2-40 (ATCC 43961) was isolated from a sample of partially degraded Spartina alterniflora at the Chesapeake Bay salt marsh in Matthews County, VA, USA (19 p.p.t. salinity). Its enzyme systems have been proposed as a model for CP degradation (Weiner, 1998; Ensor et al., 1999), and its pleomorphic morphology (Weiner et al., 1998) and tyrosinase activity (Kelley et al., 1990) have been the subject of extensive investigations.

Strain 2-40 was cultured in marine broth 2216 (Difco) with 0.2% agar. The organism is Gram-negative, pleomorphic and has a single polar flagellum (Fig. 1).

Mean cell width and length are 0.5 and 1.5–3.0 μm, respectively. In stressed cultures, filaments and coils 20 μm long are formed.

The biochemical tests (Table 1) were done as described by Smibert & Krieg (1981), except that Instant Ocean (IO; Aquarium Systems) was used instead of sodium chloride at a final concentration of 3.5%. Strain 2-40 is catalase- and peroxidase-positive and nonfermentative. Growth requires sea-salt-based medium. It grows in the mineral medium of Niven (1977), although it grows better with organic nitrogen than with ammonium. When tyrosine or peptone are present, eumelanin is produced during late phases of growth.

An ethanol-insoluble, anthrone-positive polymer accumulates when the organism is growing in glucose salts medium.

Strain 2-40 multiplies from 4 to 37 °C. The optimum pH for growth is 7.5, although it also grows at pH 4.5–10.0. The optimum sea salt concentration is 23–35 g l⁻¹, although it grows up to 100 g l⁻¹. At 60 g sea salt l⁻¹, cells become filamentous; at 80 g l⁻¹, the filamentous forms dominate and coiled cells appear; at 100 g l⁻¹, cells cling to the flask wall. Strain 2-40 does not grow below 10 g sea salt l⁻¹ and, consequently, does not grow on nutrient agar plates unless they are amended with salt.

The G + C content of the DNA, determined by thermal denaturation (Tm), is 46.7 mol%.

A number of polysaccharides were tested, as described previously (Ensor et al., 1999), for release of reducing sugars in medium inoculated with spent medium of
Fig. 1. Transmission electron micrograph of strain 2-40, grown in 0–2% glucose minimal medium to exponential phase and stained with uranyl acetate. Note the single polar flagellum. Bar, 1 µm.

Table 1. Phenotypic comparison of selected traits of marine bacterium strain 2-40 with those of its closest relative based on 16S rDNA analysis, *Microbulbifer hydrolyticus*

<table>
<thead>
<tr>
<th>Trait</th>
<th>Strain 2-40</th>
<th><em>Microbulbifer hydrolyticus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell shape</td>
<td>Pleomorphic rods</td>
<td>Rods</td>
</tr>
<tr>
<td>Cell length</td>
<td>1–20 µm</td>
<td>1–1.7 µm</td>
</tr>
<tr>
<td>Cell arrangement</td>
<td>Single, chains</td>
<td>Single, short chains</td>
</tr>
<tr>
<td>Flagellum</td>
<td>Single, polar</td>
<td>None</td>
</tr>
<tr>
<td>Surface nodular-like structures</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Growth temperature range</td>
<td>4–37 °C</td>
<td>10–41 °C</td>
</tr>
<tr>
<td>Growth pH range</td>
<td>4.5–10.0</td>
<td>6.5–8.5</td>
</tr>
<tr>
<td>Requirement for &gt;1% salt for growth</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Synthesis of eumelanin</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Growth factor requirement</td>
<td>B cofactors stimulatory</td>
<td>–</td>
</tr>
<tr>
<td>Growth on monosaccharides</td>
<td>+</td>
<td>Limited number</td>
</tr>
<tr>
<td>Hydrolysis of:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cellulose</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Agar</td>
<td>+</td>
<td>–</td>
</tr>
</tbody>
</table>

Both organisms synthesize catalase and oxidase; do not reduce nitrate; oxidize but do not ferment fructose, galactose, galacturonic acid, glucose, gluconic acid, lactose, maltose, mannose, sucrose and xylene; hydrolyze Tween 80, xylan, carboxymethylcellulose, starch and gelatin; and are sensitive to ampicillin and tetracycline.

Strain 2-40 synthesizes a true tyrosinase, forming melanin from L-tyrosine. This enzyme also acts on L-DOPA, D-tyrosine, p-cresol, catechol, 2,6-dimethoxyphenol, syringaldazine, guaiacol and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonate) (ABTS) (Kelley et al., 1990; Solano & Sanchez-Amat, 1999).

For fatty acid methyl ester (FAME) analysis, 2-40 was grown at 27 °C and harvested during the late exponential phase of growth. The FAME analysis was done using the MIDI system (Microbial ID). The following major fatty acids were detected: 16:0, 37%; 14:0, 15%; 12:1 3-OH, 6%; 10:0 3-OH, 11%; and 10:0, 5%. The major fatty acids whose levels were not quantified due to poor resolution of the chromatography system were: 18:1 ω7c, 18:1 ω9t and 18:1 ω12t. A search of the MIDI database revealed that the closest relative to 2-40 according to the fatty acid profile was *Marinobacterium georgiense* KW-40 with the following major fatty acids: 18:0, 1%; 16:0, 2-40. The following CP were positive: agar, agarose, alginic acid, arabin, carrageenan, carboxymethylcellulose, chitin, fucoidan, glycogen, laminaran, pectin, pullulan, sodium polygalacturonate, starch and xylan. The following compounds were negative after 2 d incubation at 30 °C: cellulose, inulin, polygalacturonic acid and Sephadex.

Methods used for antibiotic sensitivity testing were described previously (Weiner et al., 1985). Strain 2-40 was resistant to bacitracin (0.04 U), erythromycin (5 µg), nitrofurantoin (300 µg), P Taxo (5 µg), penicillin G (10 U), polymyxin (300 U) and streptomycin (2 µg), and susceptible to ampicillin (10 µg), novobiocin (30 µg), sulfadiazine (300 µg) and tetracycline (30 µg).

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Strain 2-40 is one of two marine isolates, the other being *Marinomonas mediterranea* MMB-1, which are reported to express polyphenol oxidase activity. *Marinomonas mediterranea* and strain 2-40 are, however, phylogenetically distant. Polyphenol oxidase shares properties with fungal laccases, enzymes known to be involved in lignin degradation (Solano & Sanchez-Amat, 1999). Therefore, the polyphenol oxidases of the two bacteria may be involved in the degradation of lignin in the aquatic environment.

It is significant that the 16S rDNA sequence of 2-40 is most closely affiliated with the 16S rDNA sequences of *Microbulbifer hydrolyticus* and a symbiont of shipworms. Several bacterial isolates were obtained in association with six different strains of shipworm and possibly other species of teredinids. The symbionts were proposed to be the same species of bacteria based on physiological and metabolic characteristics (Waterbury et al., 1983) and this was later confirmed by sequence analysis of the 16S rDNA (Distel et al., 1991). Although there is not yet enough data to ascertain the taxonomy of the shipworm isolate, it may belong to the genus *Microbulbifer*. Thus, *Microbulbifer hydrolyticus*, 2-40 and the shipworm symbionts form a cluster of marine bacteria with strong and extensive ability to degrade CP and other polymers of plant and animal origin. Both strain 2-40 and *Microbulbifer hydrolyticus* also express vesicular or nodular-like structures on the outer membrane under certain growth conditions. It is possible that, as new taxa are found that cluster with strain 2-40, this bacterium and the shipworm symbionts could be assigned to two new species of the genus *Microbulbifer* or of a closely related new genus.

### Acknowledgements

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### References


27%; 12:0, 2%; 10:0 3-OH, 6%; and 10:0, 3%. The fatty acid profile of strain 2-40 had a low level of similarity with that of *Microbulbifer hydrolyticus* IRE-31 (González et al., 1997), the closest strain according to its 16S rDNA sequence.

The 16S rDNA sequence was obtained as described by González et al. (1997) and phylogenetic analysis was done with the PHYLIP package (Felsenstein, 1989), using Jukes–Cantor corrections. It was preliminarily suggested that 2-40 could be assigned to the genus *Aleromonas* (Andrykovitch & Marx, 1988). However, the closest related sequences in GenBank were those of *Microbulbifer hydrolyticus* (93% similarity) and a cellulosytic nitrogen-fixing bacterium from the gland of Deshayes in three different species of shipworm (91.2%; Distel et al., 1991). Other closely related sequences were *Marinobacter hydro-

carbonoclasticus*, *pseudomonads*, *Oceanospirillum*, *Marinobacterium*, *Marinomonas* and members of the family *Halomonadaceae* (Fig. 2).

![Phylogeny of strain 2-40](image-url)

**Fig. 2.** Phylogeny of the 16S rRNA of strain 2-40 and representatives of the closest groups in the γ-Proteobacteria. The following sequences were used: strain 2-40, (GenBank accession no. AF052629); shipworm symbiont ATCC 39867 (M64339); *Acinetobacter calcoaceticus* ATCC 23055T (Z93434); *Agrobacterium tumefaciens* ATCC 23308T (D01257); *Halomonas meridiana* DSM 5425T (M93356); *Halomonas* (previously *Halovibrio*) variabilis DSM 3051T (M93357); *Marinobacter hydrocarbonoclasticus* ATCC 49840T (X67022); *Marinobacterium georgense* ATCC 700074T (US8339); *Marinomonas mediterranea* ATCC 700492T (AF063027); *Marinomonas vaga* ATCC 27119T (X67025); *Microbulbifer hydrolyticus* ATCC 700072T (US8338); *Oceanospirillum linum* ATCC 11336T (M22365); *Pseudomonas aeruginosa* DSM 12427T (Z76651); *Pseudomonas fluorescens* DSM 50090T (Z76662); and *Pseudomonas putida* DSM 291T (Z76667). The dendrogram was constructed by analysis of approximately 750 bp of the sequence using the neighbour-joining method (Saitou & Nei, 1987). The numbers indicate bootstrap values greater than 50%. The tree was unrooted with *Agrobacterium* *tumefaciens* as outgroup. Bar indicates Jukes–Cantor distance.

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