Saccharophagus degradans gen. nov., sp. nov., a versatile marine degrader of complex polysaccharides

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Gammaproteobacteria belonging and related to the genus *Microbulbifer* are an emerging group of complex carbohydrate-degrading marine bacteria. Previously, all of the representatives were placed within *Microbulbifer* or were unclassified. Recently, a new genus, *Teredinibacter*, represented by a single species, *Teredinibacter turnerae*, was formed to include an endosymbiotic branch of these organisms. In this study, based on 16S rRNA gene sequence similarity and phenotypic analyses, a new genus, *Saccharophagus*, is proposed to accommodate the most versatile marine carbohydrate degrader yet identified, *Saccharophagus degradans* gen. nov., sp. nov. 2-40^T (=ATCC 43961^T=DSM 17024^T). *S. degradans* strain 2-40^T can degrade 10 tested complex polysaccharides: agar, alginate, chitin, cellulose, fucoidan, laminarin, pectin, pullulan, starch and xylan. *S. degradans* 2-40^T shares 90.5% 16S rRNA gene sequence similarity with the type strain of the *Microbulbifer* type species, *Microbulbifer hydrolyticus* IRE-31^T, and 91.5% with *T. turnerae* T7902^T, and can be further distinguished from members of these two genera by 16S rRNA gene cluster analysis, the ability to utilize 10 different complex polysaccharides as sole carbon sources, a significantly lower G+C content and differences in fatty acid content. The three genera of complex polysaccharide-degrading, marine bacteria now encompass 20 strains from diverse marine niches.

The carbon cycle in marine habitats has not yet been elucidated, as many of the micro-organisms that degrade complex polysaccharides (CPs), especially those derived from higher plants and algae, have not been identified. These include highly specialized micro-organisms that recycle CP, a critical step in the marine food web. Within the past decade a number of such bacteria have been discovered.

In 1997, a Gram-negative bacterium that degrades cellulose, xylan and chitin was isolated by Gonzalez *et al.* (1997) from a salt marsh in Georgia, USA, and named *Microbulbifer hydrolyticus* IRE-31^T. In 2002, Distel *et al.* (2002) reported taxonomic criteria for a shipworm symbiont that degrades cellulose and found them to be sufficiently different from

the *Microbulbifer* taxon to be accorded a new genus, *Teredinibacter*. Within the past 2 years, sequences from six strains related to *Microbulbifer* have been deposited in the GenBank database.

In 1986, Andrykovitch & Marx (1988) isolated bacteria involved in the degradation of a salt marsh grass, *Spartina alterniflora*, found in the lower Chesapeake Bay, USA. One of these was designated strain 2-40^T. Based upon phenotypic characteristics, it was placed with the alteromonads, where it resides today in the order 'Alteromonadales' of the 'Gammaproteobacteria', in the family Alteromonadaceae. Later, based on sequence analysis of the 16S rRNA gene, its distinctive cellular morphology and its CP-degradative capabilities, 2-40^T was considered to be closely related to the genus *Microbulbifer*. A more meaningful classification of strain 2-40^T had been hampered by the limited availability of 16S rRNA gene sequences from closely related organisms. Now, on the basis of more than 20 available sequences, we propose a new genus, *Saccharophagus*, to accommodate the most versatile marine carbohydrate degrader yet identified.

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Abbreviation: CP, complex polysaccharide.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 2-40^T is AF055269.

Strain 2-40^T can degrade at least 10 CPs: agar, alginate, chitin, cellulose, fucoidan, laminarin, pectin, pullulan, starch and xylan (Ensor *et al.*, 1999; Howard *et al.*, 2003). These CPs are derived from numerous sources including algae, land plants, crustaceans, bacteria and fungi. Because of its involvement in ocean and estuarine carbon cycles, the US Department of Energy has recently (February 2005) completed sequencing the genome of strain 2-40^T (http://genome.jgi-psf.org/draft_microbes/micde/micde.home.html; named as '*Microbulbifer degradans*'). Genomic analysis has predicted that there are >130 open reading frames that encode enzymes involved in the depolymerization of CPs. Additionally, it is likely that at least another 100–200 genes are involved in signalling, regulation and further metabolism of CPs.

Strain 2-40^T clusters with the marine CP-degrading genera. Its 16S rRNA gene sequence is most closely related to those of *M. hydrolyticus* (90.5%) and *Teredinibacter turnerae* (91.5%); it shares the ability to degrade CPs with both *M. hydrolyticus* and *T. turnerae* and it has similar cellular morphology to that of *M. hydrolyticus*, most notably the copious production of membrane blebs and vesicles when grown with a CP as the sole carbon source (Gonzalez & Weiner, 2000) (Fig. 1). It is proposed that these organisms form a cluster of Gram-negative marine gammaproteobacteria with the ability to degrade CPs. The question remained whether strain 2-40^T is sufficiently different from members of these genera to warrant its placement in a new genus.

Fig. 1. Scanning electron micrograph of a late stationary phase cell of strain 2-40^T grown on glucose as the sole carbon source, viewed at 50 000 $\times$ magnification. Cells were washed in PBS, fixed in 2% glutaraldehyde, collected on 0.2 μ m filters and post-fixed in 2% OsO₄. Following dehydration in a standard ethanol series, the samples were critical-point-dried in CO₂, mounted on SEM stubs and coated with gold–palladium alloy to a final thickness of approximately 15 nm and viewed on a Hitachi S-4700 UltraHigh Resolution scanning electron microscope. Note the irregular surface topology, typical of growth on CP and in late phases of growth on glucose. Bipolar nodes, fibrils and surface protuberances, some spherical and others irregular in shape, are present. Bar, 600 nm. By contrast, a cell growing exponentially in glucose marine medium has a typical bacillus morphology (0.8 $\times$ 2.0 μ m) with a smooth surface and a single polar flagellum (Gonzalez & Weiner, 2000; not shown).

Since the initial report of strain 2-40^T (Gonzalez & Weiner, 2000), 18 additional 16S rRNA gene sequences of related organisms have become available in GenBank. These permitted clarification of the phylogenetic position of strain 2-40^T when considered along with available phenotypic analyses. The 16S rRNA gene sequence of strain 2-40^T was obtained from GenBank (accession no. AF055269). The 16S rRNA gene tree (Fig. 2) was generated using CLUSTAL W 1.82 (Chenna *et al.*, 2003) for sequence alignment and the neighbour-joining program in the PHYLIP package (Felsenstein, 1988). Before analysis, a filter was applied to exclude positions with less than 50% conservation within the sequences being aligned. Only positions 110–1265 (*Escherichia coli* numbering) were considered. All additional sequences were obtained from the GenBank database. It should also be noted that a metagenomic study of Sargasso Sea prokaryotes revealed putative proteins attributed to isolate *Microbulbifer* SAR-1 (<http://www.ncbi.nlm.nih.gov/Web/News/str/Spring04/sargasso.html>). However, 16S rRNA gene sequences from the Sargasso Sea shared <90% similarity with any isolate included in Fig. 2 and so are not included in the proposed *Microbulbifer*/*Teredinibacter*/*Saccharophagus* group.

The inferred position of strain 2-40^T falls outside of two branches of the tree (Fig. 2). The first is a *Microbulbifer*

Fig. 2. Phylogenetic position of strain 2-40^T based on its 16S rRNA gene sequence. The tree was generated using CLUSTAL W 1.82 (Chenna *et al.*, 2003) for sequence alignment and the neighbour-joining program in the PHYLIP package (Felsenstein, 1988). *Burkholderia cepacia* served as the outgroup (not shown). Numbers at nodes indicate percentage bootstrap values above 50 (100 replicates). Bar, Jukes–Cantor evolutionary distance of 0.05.

cluster that contains seven members, including 'Pelagibacter variabilis' (Imamura *et al.*, 1997). This cluster is supported by a high bootstrap score. Six members were isolated from aquatic marine habitats, while 'Microbulbifer arenaceus' was isolated within coastal sandstone (Tanaka *et al.*, 2003). The type species of the genus *Microbulbifer* is *M. hydrolyticus* (type strain IRE-31^T).

The second branch is more diffuse and contains organisms from diverse marine niches, predominantly endosymbionts. Only one of these, *T. turnerae*, has been given a validly published name (Distel *et al.*, 2002). *T. turnerae*, LP1, LP2, LP3 and AF102866 are endosymbionts of shipworms from the bivalve family Teredinidae, while 'Candidatus Endobugula glebosa' and 'Candidatus Endobugula sertula' were isolated from the pallial sinuses of the bivalve *Bugula neritina* (Haygood & Davidson, 1997; Lim & Haygood, 2004). Strain NKB4 was isolated from deep-sea sediments (Li *et al.*, 1999b). The 16S rRNA gene sequences of strains SCB11 and BD2-13 were derived gene clones from marine environmental samples (Li *et al.*, 1999a). Strain characteristics for these organisms have not been reported.

Strain 2-40^T shares a maximum of 91 % 16S rRNA gene sequence similarity with members of the genus *Microbulbifer* and 93 % similarity with the endosymbiont cluster. It shares 91.9 and 91.3 % similarity with BD2-13 and SCB11, respectively. These data suggest that strain 2-40^T is related to the other members included in the 16S rRNA gene tree, but

below the species level. This is in agreement with previously published data (Distel *et al.*, 2002; Gonzalez & Weiner, 2000; Tanaka *et al.*, 2003; Yoon *et al.*, 2003a).

The suggestion that strain 2-40^T is distinct from the other *Microbulbifer*/*Teredinibacter* isolates is also supported by comparison of phenotypic characteristics (Table 1). Most notably, the G + C content of strain 2-40^T is significantly lower than that estimated for the other organisms, 46.7 mol% as reported by Gonzalez & Weiner (2000) and 45.8 mol% as determined from the draft genomic sequence, compared with 57.7 mol% for *M. hydrolyticus* and 49–51 mol% for several *T. turnerae* strains (Distel *et al.*, 2002). Furthermore, the major fatty acid of strain 2-40^T is iso-C_{16:0} (37 %) (Gonzalez & Weiner, 2000), while iso-C_{15:0} is the primary fatty acid of *M. hydrolyticus* (Gonzalez *et al.*, 1997) and *Microbulbifer salipaludis* (Yoon *et al.*, 2003a). Strain 2-40^T reduces nitrate to nitrite and synthesizes eumelanin via tyrosinase activity (Kelly *et al.*, 1990), both traits reported for only one other strain, 'M. arenaceus' (Tanaka *et al.*, 2003), which does not degrade agar and shares only 90.6 % 16S rRNA gene sequence similarity with strain 2-40^T.

Strain 2-40^T was known to degrade at least 10 CPs, many more than had been reported for *T. turnerae* and strains of *Microbulbifer* (Table 1). However, because this required additional confirmation, the differences in CP-degradative capabilities within the *Microbulbifer*/*Teredinibacter*/*Saccharophagus* group of bacteria were investigated.

Table 1. Distinguishing characteristics of strain 2-40^T compared with *Microbulbifer* species and *T. turnerae*

Species/strains: 1, strain 2-40^T [data from Gonzalez & Weiner (2000) unless indicated]; 2, *M. hydrolyticus* (Gonzalez *et al.*, 1997); 3, *M. salipaludis* (Yoon *et al.*, 2003a); 4, *M. elongatus* (Yoon *et al.*, 2003b); 5, *Microbulbifer maritimus* (Yoon *et al.*, 2004); 6, 'M. arenaceus' (Tanaka *et al.*, 2003); 7, *T. turnerae* (Distel *et al.*, 2002). Substrates were tested for depolymerization and utilization; +, substrate depolymerized; –, substrate not depolymerized under conditions tested. ND, Not described.

Characteristic	1	2	3	4	5	6	7
G + C content (mol%)	45.8	57.7	59	ND	59.9	ND	49–51
Major fatty acid	iso-C _{16:0}	iso-C _{15:0}	iso-C _{15:0}	iso-C _{15:0}	iso-C _{15:0}	ND	ND
Eumelanin production	+	–	–	–	–	+	–
Nitrate reduction*	+†	–†	ND	–†	ND	+	ND
Gelatin utilization*	–†	+†	ND	+†	ND	ND	ND
Substrate							
Agar	+	–	+	+	–	–	–
Alginate	+	+†	+†	+	ND	ND	ND
Cellulose	+	+	–†	+	ND	ND	+
Chitin	+	+	–	+	–	+	ND
Fucoidan	+	ND	ND	ND	ND	ND	ND
Laminarin	+	ND	ND	ND	ND	ND	ND
Pectin	+	+†	–†	+†	ND	ND	+
Pullulan	+	+†	+†	+†	ND	ND	ND
Starch	+	+	+	+	ND	+	ND
Xylan	+	+	+	–†	ND	ND	+

*Using API 20NE test strips.

†Determined in this study.

M. hydrolyticus, *Microbulbifer elongatus* and *M. salipaludis* were tested for their abilities to degrade alginate, cellulose, pectin, pullulan and xylan compared with strain 2-40^T.

Assays for the depolymerization of cellulose, pullulan and xylan utilized azurine-cross-linked polysaccharides (Megazyme; <http://www.megazyme.com/>) incorporated into 1/3 strength marine agar (Difco 2216). Degradation of these insoluble substrates by endohydrolases produced soluble dye-labelled fragments that were easily observed as blue haloes around active colonies. *M. hydrolyticus* was used as a positive control for xylan and cellulose utilization. Gelatin utilization and nitrate reduction were determined by API 20NE test strips supplemented with sea salts medium (2.3 % sea salts, 0.05 % yeast extract).

To assay for growth on alginate or pectin, strains were grown in minimal sea salts liquid medium incorporating the polysaccharide of interest (0.2 % final concentration). The disappearance of the polysaccharides and a reduction of broth viscosities indicated the degradation of alginate and pectin. Negative controls included media without polysaccharide. Strain 2-40^T was used as a positive control. The results are summarized in Table 1.

Strain 2-40^T depolymerized all the tested substrates shown. Each of the other related strains was negative for at least one of the substrates, even considering the fact that not every strain was available. The versatility of strain 2-40^T CP degradation is unique among the other strains of the *Microbulbifer*/*Teredinibacter*/*Saccharophagus* group and even among any other known bacteria. We believe that the phylogenetic and phenotypic analyses presented here, together with other published recommendations (Distel *et al.*, 2002; Solano & Sanchez-Amat, 1999), support the classification of strain 2-40^T as the type strain of *Saccharophagus degradans* gen. nov., sp. nov.

Description of *Saccharophagus* gen. nov.

Saccharophagus (Sac'cha.ro.pha'gus. Gr. n. *saccharon* sugar; N.L. masc. n. *phagus* from Gr. masc. n. *phagos* glutton; N.L. masc. n. *Saccharophagus* sugar-devourer).

Gram-negative, motile, heterotrophic, pleomorphic, rod-shaped, aerobic, catalase-positive and oxidase-positive. Numerous cell surface blebs and vesicles are produced. Degrades numerous complex polysaccharides. Requires sea salts for growth. The type species is *Saccharophagus degradans*.

Description of *Saccharophagus degradans* sp. nov.

Saccharophagus degradans (de.gra'dans L. part. adj. *degradans* bringing back into the former order, used to refer to the ability of the type strain to degrade several complex carbohydrates).

Cells are pleomorphic rods, averaging 1.5–3.0 µm long and

0.5 µm wide during the exponential phase of growth in glucose. In media containing complex polysaccharides as sole carbon sources, cells can be pleomorphic and produce surface protuberances and vesicles. Cells form coils and filaments when grown at high salinity. Colonies are cream in colour and then turn black upon eumelanin production. Colonies rapidly pit agar plates. Capable of utilizing the following complex carbohydrates as sole carbon sources: agar, alginate, chitin, cellulose, fucoidan, laminarin, pectin, pullulan, starch and xylan. Hydrolyses tyrosine. Temperature range for growth is 4–37 °C with an optimum of 30 °C. Optimum pH for growth is 7.5 with a range of 4.5–10. Requires sea salts for growth in the range 1–10 % with an optimum of 3.5 %. Secretes proteases. The G + C content of the type strain is 45.8 mol% as determined by genomic sequencing.

The type strain (and only strain to date) is 2-40^T (= ATCC 43961^T = DSM 17024^T). It was isolated from the surface of degrading salt marsh cord grass, *Spartina alterniflora*, in the lower Chesapeake Bay, Mathews County, VA, USA.

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