Sagittula stellata gen. nov., sp. nov., a Lignin-Transforming Bacterium from a Coastal Environment

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A numerically important member of marine enrichment cultures prepared with lignin-rich, pulp mill effluent was isolated. This bacterium was gram negative and rod shaped, did not form spores, and was strictly aerobic. The surfaces of its cells were covered by blebs or vesicles and polysaccharide fibrils. Each cell also had a holdfast structure at one pole. The cells formed rosettes and aggregates. During growth in the presence of lignocellulose or cellulose particles, cells attached to the surfaces of the particles. The bacterium utilized a variety of monosaccharides, disaccharides, amino acids, and volatile fatty acids for growth. It hydrolyzed cellulose, and synthetic lignin preparations were partially solubilized and mineralized. As determined by 16S rRNA analysis, the isolate was a member of the α subclass of the phylum *Proteobacteria* and was related to the genus *Roseobacter*. A signature secondary structure of the 16S rRNA is proposed. The guanine-plus-cytosine content of the genomic DNA was 65.0 mol%. On the basis of the results of 16S rRNA sequence and phenotypic characterizations, the isolate was sufficiently different to consider it a member of a new genus. Thus, a novel genus and species, *Sagittula stellata*, are proposed; the type strain is E-37 (= ATCC 700073).

Lignin degradation in salt marsh ecosystems is an important biogeochemical process due to the high primary productivity in such ecosystems and the abundance of vascular-plant-derived lignocellulosic material (4, 24). While both bacteria and fungi can be involved in the degradation of lignin (54), in aquatic environments bacteria are probably responsible for the utilization of the most refractory components (27, 35). In a salt marsh, bacteria mediate most of the lignin degradation (5). Although members of at least one genus of bacteria known to be involved in lignin degradation in soils (the genus *Streptomyces*) have been identified in salt marsh sediments (37), little else is known about the identities of the bacterial lignin degraders in these systems. Thus, the isolation of lignolytic bacterial strains from salt marshes is significant from an ecological perspective. In addition, waste from the pulp and paper industry is another important source of polymeric and highly recalcitrant lignin in some coastal marine environments. Microbial communities are known to mineralize this waste in salt marsh ecosystems (33).

In order to study the lignolytic potential of marine organisms, bacteria were isolated from a community growing in seawater with the high-molecular-weight fraction of pulp mill effluent as the sole carbon source. One isolate, strain E-37^T, became a dominant member of this enrichment community and eventually contributed up to 32% of the community DNA (22). The phenotypic and phylogenetic characteristics of this isolate indicate that it belongs to a new genus of marine bacteria. In this paper we propose the name *Sagittula stellata* gen. nov., sp. nov., and designate strain E-37 the type strain of this species.

MATERIALS AND METHODS

Isolation. Strain E-37^T was isolated on YTSS medium (see below) from an enrichment community culture containing the high-molecular-weight fraction (molecular weight > 1,000) of pulp mill effluent as the sole carbon source (21). The original source was seawater from the coast of Georgia.

Media and culture conditions. YTSS contained 4 g of yeast extract (Difco Laboratories, Detroit, Mich.), 2.5 g of tryptone (Difco), 20 g of sea salts (Sigma Chemical Co., St. Louis, Mo.), 18 g of agar, and 1 liter of distilled water. Other media used were marine agar 2216, marine broth 2216 (Difco), and marine salts basal medium (BM) (2) supplemented with various carbon sources (21). Unless specified otherwise, each carbon source was added at a concentration of 0.1% (wt/vol or vol/vol). BM agar was prepared by adding 18 g of agar per liter. Liquid cultures were grown at 25°C in the dark in a rotatory shaker at 300 rpm. Plate cultures were grown at room temperature.

Morphological, biochemical, and physiological tests. Routine tests, including tests for growth on different carbon sources, bacteriochlorophyll *a* production, poly- β -hydroxybutyrate accumulation, and other characteristics, were performed as described previously (21). Spore production was determined in BM containing yeast extract at the late exponential phase by heating the culture medium at 80°C for 10 min, after which the culture was serially diluted in the same medium and incubated at room temperature. Endospore formation was also examined by using the Schaeffer-Fulton staining method (38) with *Bacillus subtilis* as a positive control. Sulfite oxidation was tested in cell extracts by using ferricyanide as an artificial electron acceptor (28). The cells used for this experiment were grown in BM containing acetate, and a solution of filter-sterilized Na₂SO₃ was added to a final concentration of 20 mM when the cells were still in the exponential phase. Cells of an isolate very closely related to *Sulfobacter pontiacus* were used as a positive control. The 16S rRNA sequence of this isolate, strain EE-36 (22), exhibited 99.7% similarity to the ribosomal DNA (rDNA) sequence of *Sulfobacter pontiacus* ChLG 10. The use of Na₂SO₃ as an electron donor was also tested in BM containing decreasing concentrations of acetate (from 20 to 2 mM) and increasing concentrations of Na₂SO₃ (from 2 mM to 20 mM). The turbidity of each culture at 540 nm was compared with the turbidity of the control (medium without Na₂SO₃).

Attachment to particles was studied in BM supplemented with different carbon sources and 0.1% lignocellulose powder, birchwood xylan (Sigma), cellulose powder (ICN Biomedicals, Inc., Costa Mesa, Calif.), or glass beads. Lignocellulose was extracted from the salt marsh cord grass *Spartina alterniflora* as described by Benner et al. (3).

Sequencing of 16S rDNA. The following oligonucleotide primers were used for sequencing (*Escherichia coli* numbering system): 19F (CTGGCTCAGARC GAACG) (34); 68F (TNANACATGCAAGTCGAKCG); 338F (ACTCCTACG GGAGGCAGC); 338R (GCTGCCTCCCGTAGGAGT) (50); 489R (GCCGG GGTTCCTTACCA) (MALF-1; see below); 536R (GWATTACCGCGCKG CTG) (31); 785F (GGATTAGATACCCNGGTA) (9); 926R (CCGTC AATTC MTTTGAGTTT); 926F (AAACTCAAAGAATTGACGG); 1406R (ACGGG CGGTGTGTRC) (31); and 1523R (AGGAGGTGATCCAGCCG) (K is G or T, R is A or G, W is A or T, M is A or C, and N is any base).

Comparative analysis of 16S rDNA. For the phylogenetic analyses, only unambiguous positions were considered (positions 60 to 1373; positions 180 to 480 when the sequences 37SW-1 and 37SW-2 were used [*E. coli* numbering system]). Parsimony and bootstrap analyses were performed as described by González et al. (21), except that the branch-and-bound method in addition to the heuristic method was used for parsimony analysis. The least-squares method of Fitch and Margoliash (15) contained in the PHYLIP package (14) was also used. Different ingroups and outgroups were used for comparison. Secondary structures were

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predicted with the help of the computer program MulFold (25, 26, 56) and were further refined by eye.

Recovery of related sequences in seawater. The recovery of 16S rDNA sequences from seawater DNA that were related to the E-37^T sequence has been described by González et al. (22). DNA was extracted from the bacterial community in the seawater that served as the original inoculum for the enrichment cultures. PCR amplification of DNA was carried out with two PCR primers, one designed specifically to target strain E-37^T (ROS137-37; 5'-TTCTGTTGAGGA TAGCCC; positions 137 to 154 [*E. coli* numbering]) and one designed to target a larger group of marine bacteria, including E-37^T (MALF-1; 5'-GCCGGGGT TTCTTTACCA; positions 488 to 505). This set of primers generated DNA fragment 37SW-1. A second PCR amplification from seawater DNA, in which we used primer ROS137-37 and a new primer designed to exclude the first sequence obtained (MALF-2; 5'-CGGGGTTCTTACCAGG; positions 486 to 503), generated DNA fragment 37SW-2.

Electron microscopy. The cells used for electron microscopy were grown in BM containing 0.2% glucose and 0.01% yeast extract. Negative staining was performed as described by González et al. (21).

DNA base composition. DNA base composition was determined by high-performance liquid chromatography (21, 36).

Cell membrane fatty acid analysis. A fatty acid methyl ester analysis was performed by workers at Microbial ID, Inc. (Newark, Del.) as described previously (21). Strain E-37^T was grown on tryptic soy broth (Difco), on which it grew weakly.

Synthetic lignin mineralization. The lignin-degrading ability of E-37^T was determined in BM containing 0.2% glucose and 0.01% yeast extract. The amount of radioactively labeled β -carbon or ring carbon dehydropolymerisate (DHP) of coniferyl alcohol added to the medium was 4,000 dpm/ml. The specific activities of [β -¹⁴C]DHP and [ring-¹⁴C]DHP were 286,000 and 434,000 dpm/mg, respectively. Radioactively labeled DHP was prepared by J. Trojanowski (University of Göttingen, Göttingen, Germany) who used the method described by Freudenberg and Neish (17). The average molecular weight of DHP was 2,330, as determined by gel permeation chromatography performed with a Sephadex G-50 gel (52). Mineralization was measured in triplicate in 30-ml portions of medium in 125-ml milk dilution bottles; uninoculated bottles were included as controls. DHP was dissolved in 10.6 ml of 0.12 M NaOH and filter sterilized before it was added to the medium. In some cases, Indulin AT (Sigma), a lignin-rich by-product of the pulping process, was added to the medium at a concentration of 0.04% (wt/vol) along with the DHP solution. Evolved ¹⁴CO₂ was trapped in 2 ml of a 0.2 M NaOH solution placed in a small vial inside each bottle. For counting, the bottles were opened and the vials were removed. A 1-ml portion of the trapping solution was added directly to 10 ml of ScintiSafe Econo 2 (Fisher Scientific, Pittsburgh, Pa.) scintillation cocktail for counting. The bottles were left open for 5 min before new NaOH solution was added and the bottles were resealed.

After 30 days of incubation, the amount of soluble lignin in the culture medium was measured. The medium was first flushed with humid air to drive off any remaining ¹⁴CO₂ and then filtered through 0.22- μ m-pore-size filters (Acrodisc; Gelman Sciences, Inc., Ann Arbor, Mich.) to remove bacteria and insoluble DHP. A 0.4-ml portion of the filtrate was counted in 10 ml of liquid scintillation cocktail. The pH of the filtrate was adjusted to 2.5 to 3 with 10 M HCl. The solution was allowed to stand overnight to allow precipitation of the remaining DHP and any acid-insoluble products, filtered as described above, and neutralized with 10 M NaOH (53). The radioactivity in the filtrate was counted as described above.

Nucleotide sequence accession numbers. The 16S rRNA sequences of strain E-37^T and PCR products 37SW-1 and 37SW-2 have been deposited in the GenBank database under accession no. U58356, U58354, and U58355, respectively. The accession numbers for the sequences of the organisms used to construct phylogenetic trees are as follows: Antarctic gas-vacuolate bacterium (23), U14583; dimethylsulfoniopropionate (DMSP)-degrading bacterium, L15345; *E. coli*, J01859; *Erythrobacter* sp. strain OCh 114, M59063; methanesulfonate-degrading bacterium, U62894; *Paracoccus denitrificans*, X69159; *Prionitis lanceolata* gall symbiont, U37762; *Rhodobacter capsulatus*, D16427; *Rhodobacter sphaeroides*, D16424; *Roseobacter algicola*, X78313; *Roseobacter denitrificans*, X69159; and *Roseobacter litoralis*, X78312.

RESULTS

Isolation. Isolate E-37^T was obtained from a single colony on YTSS agar. After more than five transfers on the same medium, only one type of colony was observed on plates and one type of cell was observed in wet mounts. Thus, the culture was judged to be pure.

Cellular and colonial morphology. Light microscopic examination revealed that the cells were straight rods which stained gram negative and occurred singly. The cells did not form spores. The cells tended to form rosettes and aggregates, especially during the stationary phase, when large clumps were

visible. After 1 week, colonies on complex or defined media were 0.5 mm or less in diameter, circular, convex with entire margins, and light cream colored.

An electron microscopic analysis showed that the cells of strain E-37^T were straight rods that were approximately 2.3 μ m long and 0.9 μ m in diameter. Each cell exhibited polarity; the width of one-half of the cell was greater than the width of the other half (Fig. 1A). A holdfast structure was present at the thicker cell pole. Blebs and vesicles were also observed on the cell surfaces and free in suspension. The cells were also covered by a dense network of fine fibrils that may have been polysaccharides. This capsular material was also observed by phase-contrast microscopy following negatively staining with India ink (38). Flagella were not seen attached to cells but were seen in suspension. However, the cells were not motile during exponential growth in marine broth 2216, on marine agar, or in BM containing succinate. The cells were not heat resistant, and endospores were not observed when cultures were examined by electron or light microscopy.

Culture and growth conditions. Isolate E-37^T did not produce diffusible pigments on marine agar 2216 or BM containing yeast extract. It grew in Shioi's marine medium, as modified by Shiba (45), but did not grow on tryptic soy agar. The best growth occurred in marine broth 2216 or BM containing Casamino Acids. Strain E-37^T had an absolute requirement for NaCl and failed to grow when NaCl was replaced with KCl or LiCl. Cells lysed in distilled water if they were first washed with 0.5 M NaCl. Washing with 0.05 M MgCl₂ prevented lysis (29, 40).

Growth in BM containing glucose, acetate, citrate, or glutamate without either a vitamin solution or 0.005% yeast extract was slow. Growth was enhanced by the addition of a combination of biotin, folic acid, pyridoxine-HCl, riboflavin, thiamine, nicotinic acid, pantothenic acid, cyanocobalamin, and *p*-aminobenzoic acid. When each of these vitamins was added singly, growth was not stimulated. The addition of a combination of thiamine, nicotinic acid, pantothenic acid, and biotin had no effect. In the presence of vitamins or 0.005% yeast extract, growth was enhanced by vigorous shaking.

Similar growth rates and final cell densities were obtained in BM containing yeast extract and 0.1 $\times$, 0.25 $\times$, 0.5 $\times$, and 1 $\times$ sea salts. The temperature range for growth was 4 to 41°C. Good growth occurred at temperatures from 10 to 41°C, and the optimum temperature was 30°C (Fig. 2). At 4°C, the growth rate was very slow, about 1.5 day⁻¹. Isolate E-37^T grew in BM containing glucose at pH values from 5.5 to 8.5, but did not grow at pH 9.5 (data not shown). The best growth occurred at pH 7.5.

Physiological and biochemical characteristics. Strain E-37^T was catalase and oxidase positive. It did not form acid from glucose, did not reduce nitrate, and failed to grow under anaerobic conditions on marine agar 2216. It did not grow on plates containing BM supplemented with glucose without a source of fixed nitrogen. Although KNO₃ or Casamino Acids could substitute for NH₄Cl, considerably better growth was obtained with NH₄Cl as the nitrogen source. Good growth was also obtained when L-tryptophan was the sole carbon and nitrogen source. In the presence of NH₄Cl, the following compounds supported growth: glucose, fructose, mannose, cellobiose, xylose, glycerol, pyruvate, formate, acetate, propionate, butyrate, succinate, malate, DL- β -hydroxybutyrate, citrate, methanol, ethanol, 2-propanol, 1-butanol, L-alanine, L-arginine, L-aspartate, L-asparagine, L-glutamate, L-glutamine, L-histidine, L-leucine, L-phenylalanine, L-proline, L-serine, L-tryptophan, *N*-acetyl-D-glucosamine, benzoate, *p*-hydroxybenzoate, *p*-coumarate, cinnamate, ferulate, and vanillate. The

FIG. 1. Ultrastructure of isolate E-37^T as revealed by transmission electron microscopy of negatively stained samples. (a) Cell covered by polysaccharide fibrils (P) aggregated into bundles. The cell exhibits polarity; the right half has a greater diameter than the left half and has a holdfast structure (H) at the cell pole; blebs (B) and vesicles (V) are close to the cell surface. Bar = 0.2 μ m. (b) Polysaccharide fibrils attached to the cell surface and the fibrillar components of the holdfast structure (FH). CM, cytoplasmic membrane. Bar = 0.1 μ m. (c) Dense network of polysaccharide fibrils and vesicles in the neighborhood of the cell surface. Bar = 0.1 μ m. (d) Preparation showing detached flagellum (FL) and cell debris. Bar = 0.1 μ m.

FIG. 2. Growth response of strain E-37^T to temperature in BM containing yeast extract.

substances that were not utilized for growth included sucrose, rhamnose, lactose, 1-propanol, methylamine, benzene, toluene, phenol, salicylate, glycine, L-isoleucine, L-lysine, L-methionine, L-threonine, and L-valine. Isolate E-37^T did not hydrolyze Tween 80, chitin, gelatin, starch, birchwood xylan, or agar.

During growth on aromatic compounds, cultures of E-37^T decreased the UV absorbance of the supernatant to the point where absorbance values were near zero. Thus, these compounds were either mineralized or substantially transformed.

When the Congo red test was applied to colonies grown on Avicel, Whatman no. 1 filter paper, cotton, and carboxymethyl cellulose, clear zones were observed, which suggested that these substrates were hydrolyzed.

E-37^T was able to grow in BM containing acetate and in the presence of Na₂SO₃ up to a concentration of 20 mM. However, the cell yields, as determined from the A₅₄₀, were the same when E-37^T was grown in BM containing 2 mM acetate and in the presence and absence of 20 mM Na₂SO₃. Cell extracts of E-37^T did not oxidize sulfite when ferricyanide was the artificial electron acceptor. Under identical conditions, extracts of isolate EE-36, whose rDNA sequence was 99.7% similar to the *Sulfitobacter pontiacus* rDNA sequence, had a specific activity of 50 nmol of ferricyanide · mg of protein⁻¹ · min⁻¹.

Strain E-37^T was susceptible to ampicillin (10 µg), chloramphenicol (30 µg), kanamycin (30 µg), penicillin G (10 U), streptomycin (10 µg), tetracycline (30 µg), and vancomycin (30 µg).

Isolate E-37^T accumulated poly-β-hydroxybutyrate. It did not produce bacteriochlorophyll *a* in marine broth 2216 in the dark. Under these conditions, bacteriochlorophyll *a* was readily detected in cells of *Roseobacter denitrificans* and *Roseobacter litoralis*.

G+C content of DNA. The guanine-plus-cytosine content of strain E-37^T DNA was 65.0 ± 0.2 mol% (mean ± standard deviation; *n* = 6).

Major cellular fatty acids. Strain E-37^T contained the following major fatty acids: 16:0, 8.6%; 18:0, 6.8%; 12:1 3OH, 3.6%; and 19:0 cyclo ω8c, 1.8%. This bacterium also contained the following additional major fatty acids that were not quantitated due to poor resolution of the chromatography system: 18:1 ω7c, 18:1 ω9t, and 18:1 ω12t.

Molecular phylogenetic analysis. The sequence of approximately 1,400 bp of the 16S rRNA gene, corresponding to 97% of the 16S rRNA gene, was obtained. A parsimony and neighbor-joining analysis performed with representatives of the eubacterial and archaebacterial groups placed E-37^T in the α subclass of the *Proteobacteria*, close to the genus *Roseobacter* (levels of similarity, 93 to 94% [30, 43, 44]). The closely related sequences included the sequences of *Roseobacter algicola* (30), the heterotrophic sulfite oxidizer *Sulfitobacter pontiacus* (47,

FIG. 3. Phylogenetic position of strain E-37^T based on its 16S rRNA sequence. Representatives of the most closely related group in the α subclass of the *Proteobacteria* are included for comparison. The dendrogram was constructed by using the results of an analysis of approximately 1,300 bp of the sequence, corresponding to unambiguous positions in all of the sequences used. The tree was generated by using the neighbor-joining method contained in the PHYLIP phylogeny inference package. The numbers are the bootstrap values for each branch; the numbers in parentheses are parsimony bootstrap values (only values greater than 50% are shown). For the most part the topology was the same when we used parsimony, unweighted pair group with mathematical average, and least-squares methods; the only exception was the position of E-37^T with respect to *Sulfitobacter pontiacus*, the Antarctic bacterium, and *Roseobacter algicola*. The tree was unrooted, and *E. coli* was the outgroup. Bar = 0.05 Jukes-Cantor distance.

48), a DMSP-degrading bacterium (32), a methanesulfonate-degrading bacterium (51), an Antarctic gas-vacuolate bacterium (23), and the red alga *Prionitis lanceolata* gall symbiont, all of which were isolated from marine environments. E-37^T was most closely related to *Sulfitobacter pontiacus* (level of similarity, 95%). The levels of relatedness to other members of the α-3 subclass of the *Proteobacteria* were somewhat lower; the levels of similarity to *Paracoccus* spp. were 91 to 92%, and the levels of similarity to *Rhodobacter* spp. were less than 90% (Fig. 3 and data not shown).

Bootstrap analysis performed with the neighbor-joining and parsimony analysis revealed an unambiguous affiliation with the α-3 subclass and that strain E-37^T was more closely related to *Roseobacter denitrificans* and *Roseobacter litoralis* than to the genus *Paracoccus* or the genus *Rhodobacter*. The bootstrap values were 100% in both cases for the branches that clustered E-37^T with *Roseobacter denitrificans* and *Roseobacter litoralis* (data not shown). When other sequences in the group were considered, the position of E-37^T with respect to *Sulfitobacter pontiacus*, the Antarctic gas-vacuolate bacterium, and *Roseobacter algicola* had low bootstrap values when the neighbor-joining and parsimony methods were used. The method of analysis used resulted in different topologies and different positions for E-37^T with respect to these three sequences. Regardless of the method and sequences used, E-37^T was always placed close to the group containing *Roseobacter denitrificans*, *Roseobacter litoralis*, and the *Prionitis lanceolata* symbiont. The association of E-37^T with *Sulfitobacter pontiacus* was not robust since the method used and the different ingroups included in the analyses yielded different topologies. When the related sequences retrieved from seawater were included in the analyses, the bootstrap values were greater than 85% for the association of E-37^T with 37SW-1 and for the association of E-37^T with 37SW-2, whereas the sequence of *Sulfitobacter pontiacus*

FIG. 4. Secondary structure of helix 11 of the 16S rRNA of the α subclass of the *Proteobacteria* (55) (A) and predicted secondary structure of the corresponding region of E-37^T 16S rRNA (B). The shaded region indicates a deletion in the 16S rRNA of E-37^T. Substitutions between strain E-37^T and sequences 37SW-1 and 37SW-2 are indicated. There may be other differences in the region from position 138 to position 155 (which was covered by the upstream PCR primer). The circled base is a conserved position in the α subclass that is different in E-37^T, 37SW-1, and 37SW-2. The numbers indicate base positions in *E. coli*. The helix numbers are the helix numbers used by Dams et al. (12).

did not exhibit any association with these three sequences (data not shown).

A 16S rRNA secondary structure in the region from position 180 to position 220 is characteristic of the α subclass of the *Proteobacteria* (55). Strain E-37^T contained an 11-bp deletion, corresponding to positions 200 to 223, in helix 11 of this region (12). An alternative secondary structure for E-37^T is shown in Fig. 4B. Furthermore, the α -subclass signature nucleotide at position 197 differed in E-37^T rRNA. rDNA sequences 37SW-1 and 37SW-2 were closely related to E-37^T rRNA (levels of similarity, 96 and 98%, respectively). The level of sequence similarity between 37SW-1 and 37SW-2 was 98%. Both of these sequences also contained the same deletion in helix 11 as E-37^T. Thus, this deletion appears to be characteristic of a group of marine bacteria that are related to strain E-37^T. Sequence 37SW-1 also contained another substitution at position 197 (Fig. 4B).

Synthetic lignin transformation. Strain E-37^T was able to transform and partially mineralize radioactively labeled DHP. After 30 days of incubation, $3.5 \pm 0.05\%$ (mean $\pm$ standard deviation; $n = 3$) of the radiolabel originally in [β -¹⁴C]DHP (Fig. 5A) and $1.0 \pm 0.1\%$ (mean $\pm$ standard deviation; $n = 3$) of the radioactive label in [ring-¹⁴C]DHP were recovered as ¹⁴CO₂. In the uninoculated controls, no ¹⁴CO₂ was detected. At the pH of the medium used (pH 7.5), DHP is only partially soluble, and the fraction that was soluble at the beginning of the experiment was approximately 18%. In bottles inoculated with E-37^T, the solubilization of [β -¹⁴C]DHP increased to 54% after 30 days (Fig. 5B). In the uninoculated controls, only 28% was soluble after 30 days. At pH 2.5 to 3, the amount of acid-soluble DHP is an indication of the extent of transformation, since modifications to the polymeric DHP render it more soluble at low pH values. In bottles inoculated with E-37^T, the fraction of acid-soluble DHP was 51% after 30 days. In the uninoculated bottles, only 16% was soluble after 30 days. The fraction of DHP that was acid soluble at the beginning of the experiment was approximately 10%. These changes in the solubility of DHP indicate that E-37^T was able to transform DHP into compounds having lower molecular weights. When Indulin AT was also present in the medium, the extent of mineralization of [β -¹⁴C]DHP by E-37^T decreased to 2%, and the level of solubilization was not significantly different from that in the

FIG. 5. Cultures of E-37^T mineralized (A) and solubilized (B) synthetic lignin ([β -¹⁴C]DHP) after 30 days. In panel B the solid bars represent cultures, and the shaded bars represent uninoculated controls. Bars 1 and 2 indicate percentages of mineralization; bars 3 and 4 indicate percentages of soluble DHP at pH 7.5; and bars 5 and 6 indicate percentages of soluble DHP at pH 2.5 to 3. Some error bars are hidden within symbols (A) or are insignificant (B); the error bars indicate 1 standard deviation.

uninoculated controls (30% at pH 7.5). Thus, Indulin AT greatly inhibited the solubilization of DHP, possibly because of the structural similarity of the two compounds. Because mineralization was not affected as greatly, mineralization of DHP may have been due to lower-molecular-weight compounds that are also in Indulin AT but are readily utilized by the bacterium.

Attachment to lignocellulose particles. Cells of strain E-37^T attached to cellulose and *Spartina alterniflora* lignocellulose particles in the presence of a variety of carbon sources. Attachment did not occur immediately after inoculation, but began when cells were at the exponential growth phase. The attachment appeared to be at one pole (Fig. 6). Cells also formed aggregates not associated with particles. Cells did not attach to glass or birchwood xylan (data not shown), indicating that attachment was specific.

DISCUSSION

The range of catabolic activities of strain E-37^T is consistent with the numerical abundance of this organism in the pulp mill waste enrichment culture examined. The isolate was able to utilize cellulose and transform lignin, natural polymers that were present in the enrichment culture and in the salt marsh system from which the inoculum was obtained (4, 24). Strain E-37^T was also able to grow on lignin-related compounds, such as *p*-coumarate, cinnamate, ferulate, and vanillate. Methanol and other C₁ compounds were also utilized, a characteristic of other bacterial isolates capable of growing on pulp and paper industry wastes (18, 21).

Although the rate of mineralization of DHP was limited (only 3.5% after 1 month), the rate of solubilization was significant (54% after 1 month). Greater solubilization than mineralization has been reported for other lignin-degrading bacteria; many actinomycetes, for example, release soluble lignin compounds during degradation of lignocellulose (10). Other nonfilamentous bacteria are able to solubilize lignin prepara-

FIG. 6. Phase-contrast micrographs of attachment of cells of E-37^T to cellulose (A) and lignocellulose (B) particles. The cultures used were late-exponential-phase cultures in BM containing cellobiose and 0.1% cellulose (A) and in BM containing cellobiose and 0.1% *Spartina alterniflora* lignocellulose powder (B). Cells attached as short chains (large arrow) or as single cells (small arrow). Refractile bodies within cells (small arrow) are presumably polyhydroxybutyrate inclusions. Bars = 10 μ m.

tions (39), and the white rot fungus *Phanerochaete chrysosporium* completely solubilizes the lignin fraction that is not mineralized (53). It is likely that soluble compounds are the end products of lignin degradation by this organism. Solubilization allows access to the cellulosic and hemicellulosic components of lignocellulose (11, 53).

The blebs and vesicles associated with the surfaces of strain E-37^T cells are similar to those described for a number of other bacteria, including rumen bacteria (16), marine bacteria (20, 21), anaerobes (1, 49), and pathogenic organisms (8, 19). Blebs and vesicles have also been found in bacteria associated with decaying wood (13, 46). Although their function in strain E-37^T is unknown, in other bacteria these structures contain hydrolytic enzymes for the breakdown of insoluble polymeric compounds (16) or may be involved in nutrient uptake.

Strain E-37^T formed aggregates in liquid medium both in the presence and in the absence of lignocellulose or cellulose. Rosette and aggregate formation is a characteristic that has been reported for marine bacterial isolates and seawater bacterial communities (7, 42). These marine aggregates, composed of bacteria, extracellular material, and dissolved organic carbon, are often associated with algal debris and are thought to originate from bacterial attack on polymers (6, 7). In bacterial degradation of plant material and other insoluble polymers, exopolysaccharides assist in the cellular attachment to substrate (41). In strain E-37^T, aggregate formation may be mediated by the holdfast structures or fibrils.

Isolate E-37^T is a marine bacterium that is gram negative, rod shaped, and strictly aerobic. As determined by 16S rRNA analysis, this organism was unambiguously affiliated with a group of marine bacteria in the α -3 subclass of the *Proteobacteria*. Its G+C content, absence of bacteriochlorophyll *a*, and major fatty acid profile exclude strain E-37^T from *Roseobacter denitrificans* or *Roseobacter litoralis* (45). The most similar 16S rRNA sequence was that of *Sulfitobacter pontiacus* (level of similarity, 95%). However, E-37^T did not oxidize sulfite with ferricyanide as an artificial electron acceptor, a property that is characteristic of the genus *Sulfitobacter* (47, 48). In addition, *Sulfitobacter pontiacus* did not have the deletion in the region from position 180 to position 220 present in the E-37^T sequence. Sufficient phenotypic differences from all of the previously described species belonging to the α -3 group were found to support assignment of E-37^T to a new taxon.

Even though the 16S rRNA analysis revealed that strain E-37^T is phylogenetically affiliated with the α subclass, a novel secondary structure in the region of the 16S rRNA molecule from position 180 to position 220 is proposed. This region is widely conserved elsewhere in the α subclass, so the novel structure found in E-37^T may be diagnostic for the new taxon. The novel secondary structure is characteristic of strain E-37^T and two uncultured organisms that were detected by PCR amplification of DNA extracted from coastal seawater. Thus, a signature secondary structure for this region is proposed based on the similarity of the secondary structures of the three sequences.

Description of *Sagittula* gen. nov. *Sagittula* (Sa.git'tu.la. L. fem. n. *Sagittula*, small arrow, referring to the shape of the bacterium). Cells are rod shaped, gram negative, strictly aerobic, and oxidase and catalase positive. Each cell has a holdfast structure, and the cell envelope has numerous surface vesicles derived from the outer membrane. Cells form rosettes and aggregates and grow on sugars, fatty acids, and amino acids. Sea salt-based medium is required for growth. The type species is *Sagittula stellata*.

Description of *Sagittula stellata* sp. nov. *Sagittula stellata* (stel.la'ta. L. adj. *stellata*, starry). The cells of type strain E-37 are rod shaped (approximately 2.3 μ m long, and 0.9 μ m in diameter) and have numerous vesicles on their surfaces. Colonies on marine agar 2216 are light cream colored.

The temperature range for growth is 10 to 41°C, and optimal growth occurs at 30°C. The optimal pH is 7.5, and the pH range is 5.5 to 8.5. The organism is a strict aerobe and does not denitrify. It accumulates polyhydroxybutyrate. It utilizes various carbohydrates and amino acids and some aromatic compounds, such as *p*-coumarate, cinnamate, ferulate, and vanillate, and it exhibits oxidase, catalase, and cellulase activities. During growth on glucose, cells are able to partially transform synthetic lignin. Growth without vitamins or yeast extract is slow. Capsules are produced in liquid medium.

Cells are susceptible to ampicillin, chloramphenicol, kanamycin, penicillin G, streptomycin, tetracycline, and vancomycin.

The guanine-plus-cytosine content of the DNA as determined by high-performance liquid chromatography is 65.0 mol%. On the basis of its 16S rRNA sequence, E-37^T belongs to the α subclass of the *Proteobacteria* and is related to *Sulfitobacter pontiacus* and *Roseobacter* spp. The organism was isolated from a marine enrichment community that was growing on pulp mill effluent as the sole carbon source and was originally inoculated with seawater from a salt marsh on the coast of Georgia. Type strain E-37 has been deposited in the American Type Culture Collection as strain ATCC 700073.

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REFERENCES

- Antranikian, G., C. Herzberg, F. Mayer, and G. Gottschalk. 1987. Changes in the cell envelope structure of *Clostridium* sp. strain EM1 during massive production of α -amylase and pullulanase. *FEMS Microbiol. Lett.* **41**:193–197.
- Baumann, P., and L. Baumann. 1981. The marine Gram-negative eubacteria: genera *Photobacterium*, *Beneckea*, *Aleromonas*, *Pseudomonas*, and *Alcaligenes*, p. 1302–1331. In M. P. Starr, H. Stolp, H. G. Trüper, A. Balows, and H. G. Schlegel (ed.), *The prokaryotes*. Springer-Verlag, Berlin, Germany.
- Benner, R., A. E. Maccubbin, and R. E. Hodson. 1984. Preparation, characterization, and microbial degradation of specifically radiolabeled [14 C]lignocellulose from marine and freshwater macrophytes. *Appl. Environ. Microbiol.* **47**:381–389.
- Benner, R., and R. E. Hodson. 1985. Microbial degradation of the leachable and lignocellulosic components of leaves and wood from *Rhizophora mangle* in a tropical mangrove swamp. *Mar. Ecol. Prog. Ser.* **23**:221–230.
- Benner, R., M. A. Moran, and R. E. Hodson. 1986. Biogeochemical cycling of lignocellulosic carbon in marine and freshwater ecosystems: relative contributions of prokaryotes and eucaryotes. *Limnol. Oceanogr.* **31**:89–100.
- Biddanda, B. A. 1985. Microbial synthesis of macroparticulate matter. *Mar. Ecol. Prog. Ser.* **20**:241–251.
- Biddanda, B. A. 1986. Structure and function of marine microbial aggregates. *Oceanol. Acta* **9**:209–211.
- Britigan, B. E., M. S. Cohen, and P. F. Sparling. 1985. Gonococcal infection: a model of molecular pathogenesis. *N. Engl. J. Med.* **312**:1683–1694.
- Britschgi, T. B., and R. D. Fallon. 1994. PCR-amplification of mixed 16S rRNA genes from an anaerobic, cyanide-degrading consortium. *FEMS Microbiol. Ecol.* **13**:225–232.
- Crawford, D. L., A. L. Pometto III, and R. L. Crawford. 1983. Lignin degradation by *Streptomyces viridosporus*: isolation and characterization of a new polymeric lignin degradation intermediate. *Appl. Environ. Microbiol.* **45**:898–904.
- Crawford, D. L., T. M. Pettey, B. M. Thede, and L. A. Deobald. 1984. Genetic manipulation of lignolytic *Streptomyces* and generation of improved lignin-to-chemical bioconversion strains. *Biotechnol. Bioeng. Symp. Ser.* **14**:241–256.
- Dams, E., L. Hendriks, Y. Van de Peer, J.-M. Neefs, G. Smits, I. Vandenbempt, and R. De Wachter. 1988. Compilation of small ribosomal subunit RNA sequences. *Nucleic Acids Res.* **16**:r7–r173.
- Daniel, G. F., T. Nilsson, and A. P. Singh. 1987. Degradation of lignocelluloses by unique tunnel-forming bacteria. *Can. J. Microbiol.* **33**:943–948.
- Felsenstein, J. 1989. PHYLIP—phylogeny inference package (version 3.2). *Cladistics* **5**:164–166.
- Fitch, W. M., and E. Margoliash. 1967. Construction of phylogenetic trees. *Science* **155**:279–284.
- Forsberg, C. W., T. J. Beveridge, and A. Hellstrom. 1981. Cellulase and xylanase release from *Bacteroides succinogenes* and its importance in the rumen environment. *Appl. Environ. Microbiol.* **42**:886–896.
- Freudenberg, K., and A. C. Neish. 1968. *Constitution and biosynthesis of lignin*. Springer-Verlag, Berlin, Germany.
- Fulthorpe, R. R., S. N. Liss, and D. G. Allen. 1993. Characterization of bacteria isolated from a bleached kraft pulp mill wastewater treatment plant. *Can. J. Microbiol.* **39**:13–24.
- García, M. M., S. A. W. E. Becker, B. W. Brooks, J. N. Berg, and S. M. Finegold. 1992. Ultrastructure and molecular characterization of *Fusobacterium necrophorum* biovars. *Can. J. Vet. Res.* **56**:318–325.
- Gauthier, M. J., B. Lafay, R. Christen, L. Fernandez, M. Acquaviva, P. Bonin, and J.-C. Bertrand. 1992. *Marinobacter hydrocarbonoclasticus* gen. nov., sp. nov., a new, extremely halotolerant, hydrocarbon-degrading marine bacterium. *Int. J. Syst. Bacteriol.* **42**:568–576.
- González, J. M., F. Mayer, M. A. Moran, R. E. Hodson, and W. B. Whitman. 1997. *Microbulbifer hydrolyticus* gen. nov., sp. nov., and *Marinobacterium georgiense* gen. nov., sp. nov., two marine bacteria from a lignin-rich pulp mill waste enrichment community. *Int. J. Syst. Bacteriol.* **47**:369–376.
- González, J. M., W. B. Whitman, R. E. Hodson, and M. A. Moran. 1996. Identifying numerically abundant culturable bacteria from complex communities: an example from a lignin enrichment culture. *Appl. Environ. Microbiol.* **62**:4433–4440.
- Gosink, J. J., and J. T. Staley. 1995. Biodiversity of gas-vacuolate bacteria from Antarctic sea ice and water. *Appl. Environ. Microbiol.* **61**:3486–3489.
- Hodson, R. E., R. R. Christian, and A. E. Maccubbin. 1984. Lignocellulose and lignin in the salt marsh grass, *Spartina alterniflora*: initial concentrations and short-term post-depositional changes in detrital material. *Mar. Biol.* **81**:1–7.
- Jaeger, J. A., D. H. Turner, and M. Zuker. 1989. Improved predictions of secondary structures for RNA. *Proc. Natl. Acad. Sci. USA* **86**:7706–7710.
- Jaeger, J. A., D. H. Turner, and M. Zuker. 1989. Predicting optimal and suboptimal secondary structure for RNA. *Methods Enzymol.* **183**:281–306.
- Kaushik, N. K., and H. B. Hynes. 1971. The fate of the dead leaves that fall into streams. *Arch. Hydrobiol.* **68**:465–515.
- Kelly, D. P., and A. P. Wood. 1994. Enzymes involved in microbiological oxidation of thiosulfate and polythionates. *Methods Enzymol.* **243**:501–510.
- Laddaga, R., and R. A. McLeod. 1982. Factors affecting the lytic susceptibility of marine and terrestrial bacteria. *Can. J. Microbiol.* **28**:414–424.
- Lafay, B., R. Ruimy, C. Rausch de Traubenberg, V. Breittmayer, M. J. Gauthier, and R. Christen. 1995. *Roseobacter algicola* sp. nov., a new marine bacterium isolated from the phycosphere of the toxin-producing dinoflagellate *Prorocentrum lima*. *Int. J. Syst. Bacteriol.* **45**:290–296.
- Lane, D. J., B. Pace, G. J. Olsen, D. A. Stahl, M. L. Sogin, and N. R. Pace. 1985. Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. *Proc. Natl. Acad. Sci. USA* **82**:6955–6959.
- Ledyard, K. M., E. F. DeLong, and J. W. H. Dacey. 1993. Characterization of a DMSP-degrading bacterial isolate from the Sargasso Sea. *Arch. Microbiol.* **160**:312–318.
- Maccubbin, A. E., R. Benner, and R. E. Hodson. 1983. Interactions between pulp mill effluents and microbial populations in coastal waters and sediments. *Biodegradation* **5**:246–256.
- Manz, W., R. Amann, W. Ludwig, M. Wagner, and K.-H. Schleifer. 1992. Phylogenetic oligodeoxynucleotide probes for the major subclasses of proteobacteria: problems and solutions. *Syst. Appl. Microbiol.* **15**:593–600.
- Mason, C. F. 1976. Relative importance of fungi and bacteria in the decomposition of *Phragmites* leaves. *Hydrobiologia* **51**:65–69.
- Mesbah, M., U. Premachandran, and W. B. Whitman. 1989. Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int. J. Syst. Bacteriol.* **39**:159–167.
- Moran, M. A., L. T. Rutherford, and R. E. Hodson. 1995. Evidence for indigenous *Streptomyces* population in a marine environment determined with a 16S rRNA probe. *Appl. Environ. Microbiol.* **61**:3695–3700.
- Murray, R. G. E., R. N. Doetsch, and C. F. Robinow. 1994. Determinative and cytological light microscopy, p. 21–41. In P. Gerhardt, R. G. E. Murray, W. A. Wood, and N. R. Krieg (ed.), *Methods for general and molecular bacteriology*. American Society for Microbiology, Washington, D.C.
- Perestelo, F., M. A. Falcón, and G. de la Fuente. 1990. Biotransformation of kraft lignin fractions by *Serratia marcescens*. *Lett. Appl. Microbiol.* **10**:61–64.
- Rayman, M. K., and R. A. McLeod. 1975. Interaction of Mg^{2+} with peptidoglycan and its relation to the prevention of lysis of a marine pseudomonad. *J. Bacteriol.* **122**:650–659.
- Rogers, G. M., and A. A. W. Baecker. 1987. Theories on the degradation in wood associated with glycolix-producing bacteria. *J. Inst. Wood Sci.* **11**:78–84.
- Rüger, H.-J., and M. G. Höfle. 1992. Marine star-shaped-aggregate-forming bacteria: *Agrobacterium atlanticum* sp. nov.; *Agrobacterium ferrugineum* sp. nov., nom. rev.; *Agrobacterium gelatinovorum* sp. nov., nom. rev.; and *Agrobacterium stellulatum* sp. nov., nom. rev. *Int. J. Syst. Bacteriol.* **42**:133–143.
- Shiba, T. 1989. Taxonomy and ecology of marine bacteria, p. 9–24. In K. Harashima, T. Shiba, and N. Murata (ed.), *Aerobic photosynthetic bacteria*. Japan Scientific Societies Press, Tokyo, Japan.
- Shiba, T. 1991. *Roseobacter litoralis* gen. nov., sp. nov., and *Roseobacter denitrificans* sp. nov., aerobic pink-pigmented bacteria which contain bacteriochlorophyll *a*. *Syst. Appl. Microbiol.* **14**:140–145.
- Shiba, T. 1992. The genus *Roseobacter*, p. 2156–2159. In M. P. Starr, H. Stolp, H. G. Trüper, A. Balows, and H. G. Schlegel (ed.), *The prokaryotes*, 2nd ed. Springer-Verlag, Berlin, Germany.
- Singh, A. P., T. Nilsson, and G. F. Daniel. 1990. Bacterial attack of *Pinus sylvestris* wood under near anaerobic conditions. *J. Inst. Wood Sci.* **11**:237–249.
- Sorokin, D. Y. 1995. *Sulfitobacter pontiacus* gen. nov., sp. nov.: a new heterotrophic bacterium from the Black Sea, specialized on sulfite oxidation. *Microbiology (Engl. Transl. Mikrobiologiya)* **64**:354–365.
- Sorokin, D. Y., and A. M. Lysenko. 1993. Heterotrophic bacteria from the Black Sea oxidizing reduced sulfur compounds to sulfate. *Microbiology (Engl. Transl. Mikrobiologiya)* **62**:1018–1031.
- Specka, U., S. Spreinat, G. Antranikian, and F. Mayer. 1991. Immunocytochemical identification and localization of active and inactive α -amylase and pullulanase in cells of *Clostridium thermosulfurogenes* EM1. *Appl. Environ. Microbiol.* **57**:1062–1069.
- Stahl, D. A., and R. I. Amann. 1991. Development and application of nucleic acid probes in bacterial systematics, p. 205–248. In E. Stackebrandt and M. Goodfellow (ed.), *Nucleic acid techniques in bacterial systematics*. John Wiley & Sons, Ltd., Chichester, England.

51. **Thompson, A. S., N. J. P. Owens, and J. C. Murrell.** 1995. Isolation and characterization of methanesulfonic acid-degrading bacteria from the marine environment. *Appl. Environ. Microbiol.* **61**:2388–2393.
52. **Trojanowski, J.** Personal communication.
53. **Ulmer, D. C., M. S. A. Leisola, B. H. Schmidt, and A. Fiechter.** 1983. Rapid degradation of isolated lignins by *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **45**:1795–1801.
54. **Vicuña, R.** 1988. Bacterial degradation of lignin. *Enzyme Microb. Technol.* **10**:646–655.
55. **Woese, C. R.** 1987. Bacterial evolution. *Microbiol. Rev.* **51**:221–271.
56. **Zuker, M.** 1989. On finding all suboptimal foldings of an RNA molecule. *Science* **244**:48–52.