

## *Marinobacter hydrocarbonoclasticus* gen. nov., sp. nov., a New, Extremely Halotolerant, Hydrocarbon-Degrading Marine Bacterium

M. J. GAUTHIER,<sup>1\*</sup> B. LAFAY,<sup>2</sup> R. CHRISTEN,<sup>2</sup> L. FERNANDEZ,<sup>1</sup> M. ACQUAVIVA,<sup>3</sup>  
P. BONIN,<sup>3</sup> AND J.-C. BERTRAND<sup>3</sup>

*Institut National de la Santé et de la Recherche Médicale, Unité 303, 1 Avenue Jean Lorrain, F-06300 Nice,<sup>1</sup>  
URA 671 du Centre National de la Recherche Scientifique, Université Paris VI, F-06230 Villefranche-  
sur-Mer,<sup>2</sup> and Centre d'Océanologie de Marseille, Faculté des Sciences de Luminy,  
F-13288 Marseille Cedex 9,<sup>3</sup> France*

**On the basis of phenotypical characteristics and analysis of 16S rRNA sequence, a new species belonging to a new genus is described, and the name *Marinobacter hydrocarbonoclasticus* is proposed. This organism, isolated from Mediterranean seawater near a petroleum refinery, is a gram-negative, aerobic, rod-shaped bacterium. It grows at NaCl concentrations of 0.08 to 3.5 M and uses various hydrocarbons as the sole source of carbon and energy. Its DNA has a guanine-plus-cytosine content of 52.7 mol%. The 16S rRNA analysis shows a clear affiliation between *M. hydrocarbonoclasticus* and the gamma group of the phylum *Proteobacteria*. A close phylogenetic relationship appears among the species *Marinomonas vaga*, *Oceanospirillum linum*, *Halomonas elongata*, and *Pseudomonas aeruginosa*. Because of the impossibility of finding a single most closely related species, we suggest that this bacterium be assigned to a new genus, at least temporarily. The possibility of a revision of this status when new data appear is, however, not excluded. The type strain is *M. hydrocarbonoclasticus* SP.17 (=ATCC 49840).**

A study conducted along the French Mediterranean coast to analyze the hydrocarbon degradation potential of bacterial populations led to the isolation of numerous oil-degrading strains from seawater and sediments collected in polluted areas. A gram-negative bacterium, originally named *Alteromonas* strain SP.17, was found to degrade various liquid and solid hydrocarbons and produce large amounts of a nondialyzable bioemulsifier (1). Strain SP.17 also exhibited extreme halotolerance.

In this paper, we present the results of a phenotypic examination of the strain and a phylogenetic analysis of its 16S rRNA. Both sets of data suggest that the organism represents a new species and deserves a new genus rank. Considering the marine origin of the organism as well as its high hydrocarbonoclastic potential, we propose the name *Marinobacter hydrocarbonoclasticus*, gen. nov., sp. nov., and designate strain SP.17 (=ATCC 49840) as the type strain.

### MATERIALS AND METHODS

**Bacterial strains.** *M. hydrocarbonoclasticus* SP.17 was isolated from sediments collected in the Gulf of Fos (French Mediterranean coast, 50 km north of Marseille) at the mouth of a petroleum refinery outlet chronically polluted by hydrocarbons (1).

16S rRNA studies included three reference marine strains obtained from the American Type Culture Collection (Rockville, Md.): *Halomonas elongata* ATCC 33173, *Alteromonas haloplanktis* ATCC 14393, and *Marinomonas vaga* ATCC 27119.

All strains were maintained in marine broth 2216 (Difco Laboratories, Detroit, Mich.) (49) at -80°C before tests. Bench cultures were made on marine agar 2216 (Difco).

**Growth conditions.** *M. hydrocarbonoclasticus* was aerobically cultivated in synthetic medium (SM) containing 1.23% (wt/vol) Tris, 0.37% (wt/vol) NH<sub>4</sub>Cl, 0.62% (wt/vol) MgSO<sub>4</sub> · 7H<sub>2</sub>O, 0.15% (wt/vol) CaCl<sub>2</sub>, and 0.075% (wt/vol) KCl amended with 0.5% (wt/vol) Bacto-Peptone (Difco) and NaCl to various concentrations. The pH was adjusted to 7.5 with 10 N HCl. Growth was estimated from optical density measurements at 600 nm with a Uvikon 720LC UV-visible-light spectrophotometer (Kontrol, Paris, France).

**Electron microscopy.** Detailed cell shapes and flagella were examined both by scanning electron microscopy and transmission electron microscopy. For scanning electron microscopy analysis, bacteria were fixed with 1% glutaraldehyde in the culture medium for 2 h and filtered on a Nuclepore filter (0.22-μm pore size; Nuclepore Corp., Pleasanton, Calif.). They were then postfixed on the filter with 2% osmium tetroxide and dehydrated in ethanol. After critical-point drying, they were sputter coated with gold-palladium and observed with a Hitachi S570 electron microscope (6). For transmission electron microscopy examinations, cells were negatively stained with phosphotungstic acid according to the method of Jahn (18) and observed with a Philips CM2 transmission electron microscope at 100 kV.

**Phenotypic analysis.** Routine tests (Gram staining, oxidase, catalase, gelatinase, phosphatase, esculinase, Tween-80, lecithinase, DNase, amylase, agarase, and urease activities) were done as described by Smibert and Krieg (33).

Denitrification was determined as N<sub>2</sub>O accumulation during anaerobic growth with NO<sub>3</sub><sup>-</sup>. The medium was SM supplemented with (weight/volume) acetate (0.1%), succinate (0.1%), and nitrate (4 mM). The last step of denitrification (N<sub>2</sub>O reduction) was blocked with acetylene (10 kPa). N<sub>2</sub>O was determined by gas chromatography (7). The extraction of N<sub>2</sub>O from the liquid phase was carried out by the procedure of Chan and Knowles (10) modified with the multiple-equilibrium technique (25).

Arginine dihydrolase, lysine decarboxylase, and ornithine

\* Corresponding author.

decarboxylase activities and accumulation of poly- $\beta$ -hydroxybutyrate were determined by using the techniques described by Bauman et al. (2, 3) for marine bacteria.

Utilization of carbon sources was tested in SM without peptone supplemented with the compounds (listed below and in Table 1) at concentrations of 0.1% (wt/vol). Salt tolerance was tested in SM supplemented with peptone, acetate, or eicosane and containing appropriate concentrations of NaCl (0.08 to 3 M).

Lysis in solutions with or without Na<sup>+</sup> ions was studied with cells grown to mid-exponential phase in SM supplemented with NaCl to 0.6 M and acetate as the source of carbon and energy. Cells were washed with solutions of either NaCl (0.5 M) or MgCl<sub>2</sub> (0.05 M) and suspended in distilled water according to the procedure described by Laddaga and McLeod (23). The  $A_{450}$ s of the final suspensions were recorded with a Shimadzu spectrophotometer (1-cm light path) for 4 h.

Susceptibility to inhibitors was measured on marine agar medium by using the standard antibiogram method (9) with the following antibiotics (Bio-disks; BD-Mérieux, Marcy l'Etoile, France): penicillin G (10 U), cephalexin (30 U), oxacillin (5 U), ampicillin (10 µg), novobiocin (30 µg), kanamycin (30 µg), streptomycin (10 µg), tobramycin (10 µg), chloramphenicol (30 µg), tetracycline (30 µg), oleandomycin (15 µg), erythromycin (15 µg), lincomycin (15 µg), spiramycin (100 µg), staphylomycin (15 µg), cephaloridine (30 µg), gentamicin (10 µg), nalidixic acid (30 µg), and the vibriostatic pteridin O/129 (100 µg).

**Spectrometric technique.** Dual-wavelength spectrometry was carried out with a Johnson Foundation spectrometer (12). The recording conditions corresponded to a 4-nm band width. The transmitted light was monitored by using a fused-silica-window photomultiplier (EMI 9558QB) encompassing the complete visible range. A microprocessor-controlled unit performed datum acquisition in a scanning mode with automatic baseline correction. Absorbance variations were plotted on an XY recorder (BD90 Kipp and Zonen). The reference wavelength was 575 nm.

**DNA base composition.** The G+C content of the DNA of *M. hydrocarbonoclasticus* SP.17 was measured by the thermal denaturation method (19) on DNA extracted by following the method of Marmur (26).

**Obtaining and sequencing rRNA.** rRNA was obtained after lysis in thiocyanate guanidinium, extraction with phenol-chloroform, and ethanol precipitation with no further purification. Sequencing was done by the Sanger method modified to accommodate reverse transcriptase in place of DNA polymerase (28) and a set of primers complementary to conserved regions located along the 16S rRNA molecule.

**Phylogenetic analysis and alignment.** (i) **General procedure.** The 16S rRNA sequence of the bacterium was aligned by comparisons with about 500 other 16S rRNA sequences already aligned on the basis of their phylogenetic relationships. Such alignment of the unknown sequence was obtained first by using only the most conserved parts of the sequences (domain D2; see below and reference 40), which it was possible to align for all bacteria without ambiguity. The sequence was progressively aligned as the phylogenetic analysis of aligned domains revealed the closely related bacteria. All operations were done using computer programs developed by us and available on request from R. Christen.

(ii) **Phylogenetic analysis.** Four different methods were used to analyze the data. A neighbor-joining method (32) was always used as a preliminary analysis, since its results have been proved to be relatively robust and were very quickly

obtained on our computer (PC 386, 20 MHz). The topologies obtained were always confirmed by using a parsimony analysis, i.e., the PAUP program for the MacIntosh (38). For our data, both methods always gave similar results. Confirmation of these results were then obtained by using bootstrap analysis and Lake's method of invariants (both included in the PAUP package). Finally, we used methods such as maximum likelihood from the PHYLIP package obtained from J. Felsenstein (15).

The PAUP analysis was usually undertaken first as a heuristic search. It was followed by a branch-and-bound option and generally led to several most-parsimonious trees. These trees differed mostly in the order of branching of the most peripheral branches, and the uncertainties were resolved by using either a consensus topology or the reweighting option (see the PAUP manual [38]). The two operations led to similar topologies, which were always in agreement with the neighbor-joining analysis, at least concerning the place of strain SP.17. Additionally, searches were undertaken for trees five steps longer than the most-parsimonious tree in order to analyze whether a particular topology was robust concerning the position of strain SP.17.

Lake's method of invariants was used only for the last part of the analysis, i.e., finding the closest relatives, since only four taxa can be analyzed at a time. This approach could not be used, for example, to study the ~100 sequences known for the proteobacteria.

(iii) **Domains used.** A phylogenetic analysis of nucleic acid sequences is also a problem of alignment and of choice of the parts of the sequence to be analyzed (see reference 15, for example). To avoid the problems of multiple substitutions and sampling artifacts, we have used several domains at each step of the analysis. The following domains have been used: D2 (conserved domain [40]), D3 (proteobacteria; 50% consensus), D4 (gamma bacteria; domains of low variation), D5 (5' half of the 16S rRNA), D6 (3' half of the 16S rRNA), and D7 (entire sequence).

The 50% consensus sequences were the positions at which 50% of the sequences within a monophyletic group of bacteria showed a similar nucleotide.

**16S rRNA sequence accession number.** The 16S rRNA sequences of *M. hydrocarbonoclasticus*, *H. elongata*, *A. haloplanktis*, and *M. vaga* have been assigned the EMBL numbers X67022, X67023, X67024, and X67025, respectively.

## RESULTS

**Cell shape, Gram stain, and motility.** *M. hydrocarbonoclasticus* strain SP.17 (=ATCC 49840) is a gram-negative, rod-shaped, nonsporeforming organism. Cells actively growing in marine broth and SM-peptone-NaCl (0.6 M) at 32°C are 2 to 3 µm in length and 0.3 to 0.6 µm in diameter. They are shorter (1.2 to 1.5 µm) in SM-eicosane-NaCl (0.6 M). Cell shape and flagellation are largely affected by the NaCl content of the medium. Cells are motile by means of a single unsheathed polar flagellum in SM-peptone with intermediate NaCl concentrations (0.6 to 1.5 M) (Fig. 1C) and nonmotile (without a flagellum) in media of low ( $\leq 0.2$  M) or high ( $\geq 1.5$  M) NaCl concentrations. Numerous blebs develop at the surface of cells grown on eicosane (Fig. 1B and D).

**Culture characteristics.** On marine agar and SM-peptone-0.6 M NaCl incubated at 32°C, young colonies are circular, with diameters of 1 to 2 mm, smooth, convex, and white, with regular edges. After 1 week of incubation, colonies enlarge to 2 to 4 mm in diameter and become rosy beige

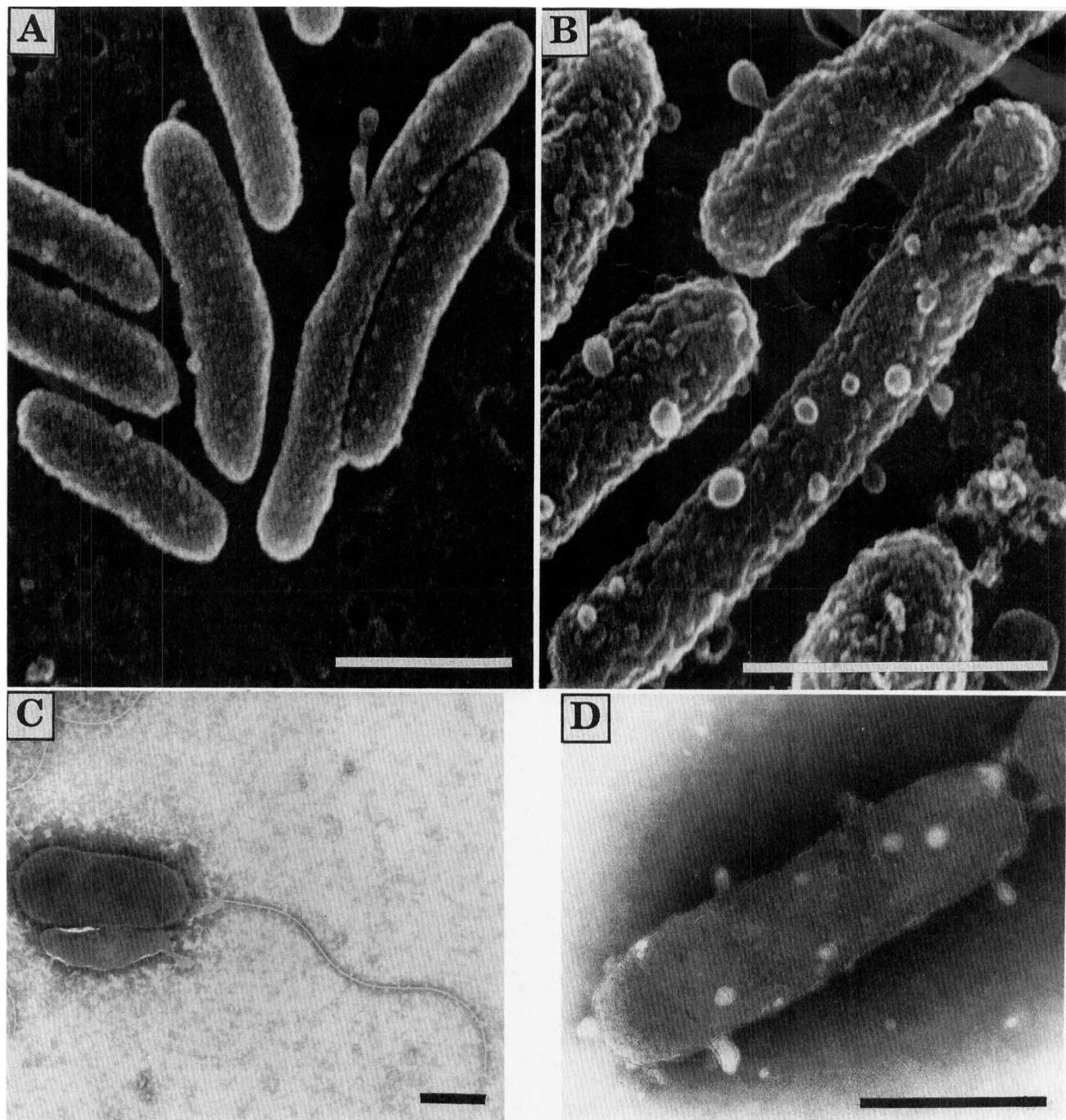


FIG. 1. Scanning (A, B) and transmission (negative staining; C, D) electron micrographs of *M. hydrocarbonoclasticus* SP.17 (=ATCC 49840) cells grown on acetate (A, C) or eicosane (B, D) as sole sources of carbon and energy at 32°C in SM containing 0.6 M NaCl. Cells were fixed or stained at the end of the exponential phase of growth. Bars equal 1  $\mu$ m. Scanning electron micrographs were made by C. Bezac.

without diffusible or fluorescent pigments. No bioluminescence can be detected. It has been previously shown that the strain produces important amounts of a nondialyzable bioemulsifier when grown on hydrocarbons as sole source of carbon and energy in liquid SM (1).

**Growth at different temperatures, pHs, and salinities; requirement for sodium; and lytic phenomenon.** SP.17 is able to grow between 10 and 45°C, with optimal growth at 32°C, and tolerates a variation of pH from 6 to 9.5 (optimum, 7 to 7.5).

Duration of both lag phase and generation time increased in the presence of increasing NaCl concentrations. This effect differed, however, with the composition of the medium (Fig. 2). Lag phase and generation time were shortened when cells were progressively inoculated through the salinity gradient (inoculation made with cells grown at the immediately lower salinity) rather than directly inoculated at each salinity with cells grown at 0.2 M NaCl. This bacterium exhibited extreme halotolerance, since it was able to grow in a medium

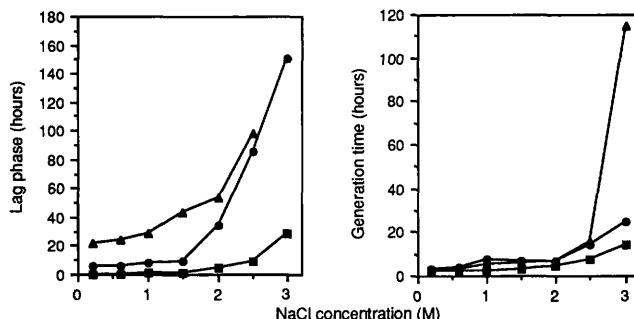


FIG. 2. Duration of lag phase of cultures of *M. hydrocarbonoclasticus* SP.17 (=ATCC 49840) and generation time of cells grown in complex medium (squares) and in SM supplemented with acetate (circles) or eicosane (triangles) as sole source of carbon and energy.

containing NaCl at concentrations ranging from 0.08 to 3.5 M. The optimal NaCl concentration for growth was about 0.6 M, which is near the NaCl concentration of Mediterranean seawater (about 0.5 M). Strain SP.17 is, then, an extremely halotolerant and slightly halophilic bacterium and can be considered a marine strain (24).

The cells had an absolute requirement for the Na<sup>+</sup> cation, since no growth was observed in a medium without Na<sup>+</sup> added, even when the osmolarity of the medium was increased by the addition of sucrose (not metabolized). However, when a constant amount of NaCl (0.1 M) was provided to cells, growth also depended on the osmotic strength of the medium (Fig. 3). The effect of a lack of Na<sup>+</sup> ions could not be reversed by the addition of LiCl or KCl, but cells grew readily when NaCl was replaced by NaNO<sub>3</sub>.

Another characteristic of strain SP.17 concerns lysis. The behavior of cells in a hypotonic medium depended on the treatment which they had previously experienced (Fig. 4). Cells washed with MgSO<sub>4</sub> solution exhibited a low level of lysis (15%) when transferred to distilled water. In contrast, the opacity of suspensions of cells washed in a 0.5 M NaCl solution showed a large decrease (58%) in distilled water. These results are in agreement with previous observations reported for other marine bacteria (5, 8, 13, 23, 30). Mg<sup>2+</sup> ions are particularly efficient in preventing lysis. We suggest that they form links between anionic groups of membrane components, such as hydrophilic-head groups of phospho-

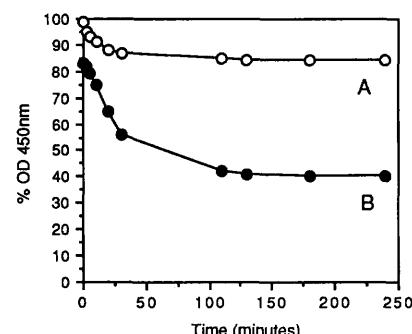


FIG. 4. Lysis of *M. hydrocarbonoclasticus* SP.17 (=ATCC 49840) cells suspended in distilled water after being washed in Tris buffer containing 0.05 M MgCl<sub>2</sub> (A) or 0.5 M NaCl (B). OD 450nm, optical density at 450 nm.

lipids and carboxyl groups of proteins. Such links should be stable enough to persist in distilled water. In the presence of a cation with a high polarizing capacity (Na<sup>+</sup>), Mg<sup>2+</sup> would be replaced by this cation (5, 11, 13, 14, 21). Cohesion of the membrane structures would then be weakened, and a further incubation of cells in distilled water would induce lysis. Such a hypothesis would account for the behavior of bacteria after washes in NaCl solution.

**Physiological and biochemical characteristics.** *M. hydrocarbonoclasticus* SP.17 exhibited oxidase, cytochrome oxidase, catalase, tweenase, and lecithinase activities. However, detailed spectrometric studies have shown a total lack of cytochrome *a-a<sub>3</sub>* oxidase and the presence of several C-type cytochromes (data not shown). After growth on alkanes (C<sub>16</sub> and C<sub>20</sub>), cells contained no cytochrome P-450 (reduced plus comminus reduced form), which has been described for different hydrocarbonoclastic microorganisms as terminal hydroxylase (29, 31, 37).

Agarase, gelatinase, amylase, urease, phosphatase, esculinase, DNase, arginine dihydrolase, lysine decarboxylase, and ornithine decarboxylase activities were not detected.

The strain is a true denitrifier, since it is able to anaerobically reduce 90% of nitrate to N<sub>2</sub>O in the presence of acetylene (39). Under anaerobic conditions, growth of *M. hydrocarbonoclasticus* occurred in the presence of KNO<sub>3</sub> as terminal electron acceptor, with citrate, acetate, or succinate (sodium salts) as sole source of carbon and energy (Fig. 5). However, growth did not occur anaerobically on glucose, with or without nitrate.

In addition to the nutritional profile shown in Table 1, strain SP.17 utilized acetate, butyrate, caproate, fumarate, adipate, DL-lactate, citrate, L-glutamate, and L-proline and was unable to utilize L-arabinose, ribose, lactose, N-acetylglucosamine, propionate, glycolate,  $\alpha$ -ketoglutarate, D-sorbitol, mandelate, benzoate, L-tryptophan, glycine, L-lysine, L-ornithine, L-asparagine, L-methionine, or sarcosine as single source of carbon and energy. The strain was also unable to utilize DL- $\beta$ -hydroxybutyrate and did not accumulate its polymer. It cleaved *p*-hydroxybenzoate but not *o*- and *m*-hydroxybenzoates. In the absence of organic nitrogen, the addition of vitamin-free Casamino Acids (Difco) markedly enhanced growth on nitrogen-free single-carbon sources. Ammonium chloride could serve as sole nitrogen source. The strain exhibited a marked hydrocarbonoclastic activity: it was able to utilize tetradecane (10%), hexadecane (100%), eicosane (91%), heneicosane (84%), pristane (34%),

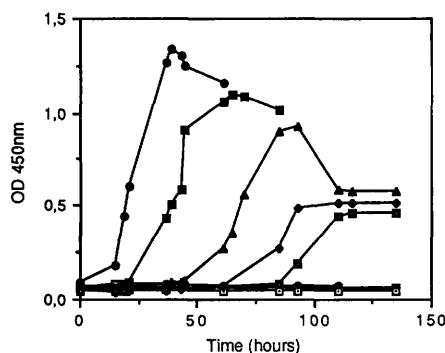


FIG. 3. Growth of *M. hydrocarbonoclasticus* SP.17 (=ATCC 49840) in SM supplemented with acetate as sole source of carbon and energy and different concentrations of saccharose (0.2, 0.6, 1, 1.5, and 2 M) with (closed symbols) or without (open symbols) NaCl (0.1 M). OD 450nm, optical density at 450 nm.

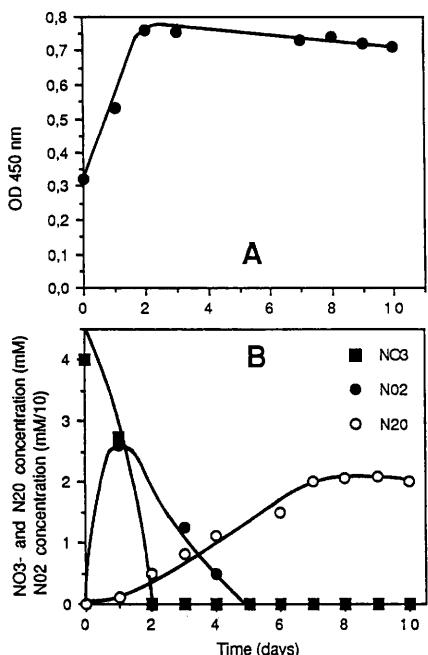


FIG. 5. Anaerobic growth of *M. hydrocarbonoclasticus* SP.17 (=ATCC 49840) in SM in the presence of nitrate (A) and evolution during increase of nitrate ( $\text{NO}_3^-$ ), nitrite ( $\text{NO}_2^-$ ), and nitrous oxide ( $\text{N}_2\text{O}$ ) concentrations in the medium (B).  $\text{N}_2\text{O}$  reduction was blocked with acetylene (as described in Materials and Methods). OD<sub>450nm</sub>, optical density at 450 nm.

phenyldecanoate (10%), and phenanthrene (41%) as single sources of carbon and energy (1).

**Resistance and susceptibility to inhibitors.** The strain is sensitive to penicillin G, ampicillin, kanamycin, streptomycin, tobramycin, chloramphenicol, erythromycin, spiramycin, cephaloridine, gentamicin, and nalidixic acid. It is totally resistant to the other tested antibiotics and to the vibriostatic pteridin O/129. For the last, however, the result is not fully reliable, since the test had to be carried out in a saline medium (27).

**G+C content of DNA.** The guanine-plus-cytosine content of the DNA is 52.7 mol%.

**Molecular phylogenetic analysis.** The phylogenetic position of strain SP.17 was examined by comparing its 16S rRNA sequence with sequences in our data bank of about 500 aligned 16S rRNA sequences of other bacteria belonging either to the archaeabacterial kingdom or to the different eubacterial phyla as defined by Woese and coworkers (16, 44–48) and Stackebrandt and coworkers (35, 36). This analysis was conducted by using several methods such as pair-wise distances, parsimony, and invariant and maximum likelihood as described in Materials and Methods.

16S rRNAs of three other previously described marine bacteria (*H. elongata*, *M. vaga*, and *A. haloplanktis*) were also sequenced for the purpose of this analysis, since our analysis based on classical methods of bacteriology (see above) indicated that strain SP.17 could be closely related to these species.

(i) **Strain SP.17 belongs to phylum Proteobacteria, gamma subgroup.** The identification of this bacterium as a eubacterium was clear (data not shown), since it showed all the molecular signatures of the eubacteria and none of the archaeobacteria. Furthermore, all phylogenetic analyses of

its 16S rRNA sequence unambiguously demonstrated that strain SP.17 belonged to the phylum *Proteobacteria*, gamma subgroup.

(ii) **Position of strain SP.17 within phylum Proteobacteria.** 16S rRNA sequences of all bacteria for which a sequence was known and that belonged to the gamma group of *Proteobacteria* were compared with the 16S rRNA sequence of strain SP.17. Their phylogenetic relationships were then analyzed by using the methods described above, and this study was repeated with several different subdomains of the 16S rRNA sequence and bootstrapping in order to avoid sampling artifacts as much as possible. Figure 6 and Table 2 summarize the results obtained. Strain SP.17 showed a rather deep branching, with much uncertainty concerning its closest affiliation. According to the boundaries chosen and the method of analysis, SP.17 was seen as closest relative to a number of bacteria that included *M. vaga*, *Oceanospirillum linum*, *Pseudomonas aeruginosa*, and *H. elongata*.

(iii) **Determination of the closest relative to strain SP.17.** In order to examine in detail whether strain SP.17 could be more closely related to one bacteria than to the others, the analysis was confined to these four most probable relatives and *Acinetobacter calcoaceticus*, as the closest outgroup (see reference 34 for a discussion of this approach).

Neighbor-joining, parsimony, maximum likelihood, and invariant (Lake's method) analyses were then undertaken on this subset of bacteria, using several subdomains of these sequences successively for the phylogenetic analysis (domains D2, D4, D5, D6, and D7; see Materials and Methods). Depending on the boundaries chosen, parsimony or neighbor joining connected strain SP.17 with different closest relatives; a loose association with *H. elongata* or *O. linum* was often obtained, but short internal branches indicated that the topology was not very robust. In parsimony, for example, one or two steps longer than the most-parsimonious tree disrupted these associations. Bootstrap analysis also revealed that these associations were extremely weak.

The analyses by maximum likelihood also showed different results according to the boundaries used, despite the fact that all branches of each tree were seen as significantly positive at  $P < 0.01$ .

This uncertainty was confirmed by using Lake's method of invariants, which showed that when these six species were analyzed according to all possible quartets (and all possible boundaries), in no case was strain SP.17 associated with any other bacterium at  $P < 0.05$ . Only in some cases was it associated with *H. elongata* at a  $P$  of 0.05 but not below.

## DISCUSSION

**Genus and species assignment.** Phenotypic characteristics, such as the absence of fermentative metabolism, a higher halotolerance, and a restricted nutritional profile, seem to be sufficient to preclude inclusion of our strain in the genera *Vibrio*, *Aeromonas*, and *Plesiomonas*. On the other hand, this strain has many traits required for inclusion in the genera *Alteromonas*, *Marinomonas*, *Oceanospirillum*, *Alcaligenes* (marine species), and *Halomonas* (Table 1), all of which group gram-negative marine species with respiratory metabolism. Its high halotolerance and vigorous denitrification would suffice to disallow inclusion in genera *Marinomonas* and *Oceanospirillum*. Species from these two genera also differ from our strain by several characteristics: absence of oxidase activity, larger nutritional profile, and ability to oxidize glucose in *Marinomonas* species; and helical structure of cells, lower temperature tolerance, and absence of

TABLE 1. Phenotypic characters differentiating *M. hydrocarbonoclasticus* from other genera and species of halophilic gram-negative bacteria<sup>a</sup>

| Characteristic                                | <i>Alteromonas</i><br>spp. <sup>b</sup> | <i>Marinomonas</i><br>spp. <sup>b</sup> | <i>Alcaligenes</i> spp.<br>(marine) <sup>c</sup> | <i>Oceanospirillum</i><br>spp. <sup>d</sup> | <i>H. elongata</i> <sup>e</sup> | <i>M. hydrocarbon-</i><br><i>oclasticus</i> |
|---|---|---|--|---|---------------------------------|---|
| Typical gram-negative cell wall profile       | +                                       | +                                       | +  | +   | +                               | +   |
| Morphology                                    | Rods                                    | Rods                                    | Rods   | Helical cells                               | Rods                            | Rods  |
| No. and arrangement of flagella               | 1, polar                                | 1, polar                                | Peritrichous                                     | Bipolar tufts                               | Peritrichous                    | 1, polar, or none                           |
| Optimal temp (°C) for growth                  | 20–25                                   | 20–25                                   | 20–25  | 25–32                                       | 30                              | 32  |
| Growth at 4°C                                 | d                                       | —                                       | —  | d   | +                               | —   |
| Growth at 45°C                                | —                                       | +                                       | d  | —   | —                               | +   |
| Optimal NaCl concn (%) for growth             | 0.7–3.5                                 | 0.7–3.5                                 |  | 0.5–8                                       | 3–8                             | 3–6   |
| Maximal NaCl concn (%) at which growth occurs |   |   |  | 9.75  | 32.5                            | 20  |
| Nitrate reduced to nitrite                    | d                                       | —                                       | d  | —   | +                               | +   |
| Nitrite reduced to N <sub>2</sub>             | d                                       | —                                       |  | —   |                                 | +   |
| Oxidase test (Kovacs)                         | +                                       | —                                       | +  | +   | +                               | +   |
| Arginine dihydrolase activity                 | —                                       | —                                       | —  |   | —                               | —   |
| Lysine decarboxylase activity                 | —                                       | —                                       |  |   | +                               | —   |
| Ornithine decarboxylase activity              | —                                       | —                                       |  |   | +                               | —   |
| Gelatin liquefaction                          | +                                       | d                                       | —  | d   | d                               | —   |
| Starch hydrolysis                             | d                                       | —                                       | —  | —   | —                               | —   |
| Urease activity                               | —                                       | —                                       | —  | —   | d                               | —   |
| Glucose oxidized                              | +                                       | +                                       | +  | —   | +                               | —   |
| Glucose fermented:                            |   |   |  |   |                                 |   |
| Without nitrate                               | —                                       | —                                       | —  | —   | —                               | —   |
| With nitrate                                  | d                                       | —                                       | —  | —   | —                               | —   |
| Utilization of:                               |   |   |  |   |                                 |   |
| D-Glucose                                     | d                                       | +                                       | +  | —   | +                               | —   |
| D-Fructose                                    | d                                       |   |  | —   | +                               | —   |
| D-Mannose                                     | d                                       | +                                       | d  | —   | +                               | —   |
| Sucrose                                       | d                                       | —                                       | d  | —   | +                               | —   |
| Celllobiose                                   | d                                       | d                                       | —  | —   | +                               | —   |
| D-Mannitol                                    | d                                       | +                                       | d  | —   | +                               | —   |
| Glycerol                                      | d                                       | +                                       | —  | —   | +                               | —   |
| D-Gluconate                                   | +                                       |   | +  |   | +                               | —   |
| Succinate                                     | d                                       | +                                       | +  | +   | +                               | +   |
| L-Glutamate                                   | d                                       | +                                       | +  | d   | +                               | +   |
| Aspartate                                     |   |   | +  | —   | +                               | —   |
| D,L-Alanine                                   | d                                       | +                                       | +  | d   | +                               | —   |
| L-Arginine                                    | d                                       | d                                       | d  | —   | +                               | —   |
| L-Serine                                      | d                                       | d                                       | d  | —   | +                               | —   |
| Hexadecane                                    | +                                       |   |  |   | +                               | +   |
| p-hydroxybenzoate                             | —                                       | +                                       | —  | d   |                                 | +   |
| Accumulation of poly-β-hydroxybutyrate        | —                                       | —                                       | +  | +   |                                 | —   |
| Mol% G+C DNA                                  | 37–50                                   | 44–48                                   | 52–68  | 42–51                                       | 60–61                           | 52.7  |

<sup>a</sup> +, positive reaction or growth; d, variable reaction; —, negative reaction or growth.<sup>b</sup> Data from Baumann et al. (4), and Gauthier and Breitmayr (17).<sup>c</sup> Data from Kersters and De Ley (20).<sup>d</sup> Data from Krieg (22).<sup>e</sup> Data from Vreeland (41–43).

poly-β-hydroxybutyrate accumulation in *Oceanospirillum* species. A phenotypical distinction from *Alcaligenes* marine species, previously described by Baumann and Baumann (3), appears more difficult. These marine bacteria, however, whose taxonomic status remained uncertain in *Bergey's Manual of Systematic Bacteriology* (20), present some distinctive differences from our strain: arrangement of flagella (peritrichous), lower halotolerance, oxidation of glucose, utilization of several carbohydrates and amino acids as sole source of carbon, and accumulation of poly-β-hydroxybutyrate.

The phenotypic characteristics of *M. hydrocarbonoclasticus* SP.17 partly conform to those given for the genus *Halomonas* by Vreeland (41): rods, generally 0.6 to 0.8 µm wide and 1.6 to 2 µm long; chemoorganotrophs capable of

respiratory but not fermentative metabolism, able to grow anaerobically on organic substrates in the presence of nitrate, and degrading hydrocarbons (hexadecane); denitrifiers; halotolerant and able to grow in a very large range of NaCl concentrations. However, many distinctive phenotypic traits of strain SP.17 differ from those of *H. elongata*, notably higher temperature tolerance, absence of anaerobic growth on glucose, and inability to utilize carbohydrates and amino acids as sole carbon source.

The G+C content of the DNA of SP.17 precludes the inclusion of this species in the genera *Alteromonas* and *Marinomonas* (which have lower G+C contents) and *Halomonas* (which has a higher G+C content) and is compatible with placing it in the genera *Alcaligenes* and *Oceanospirillum*.

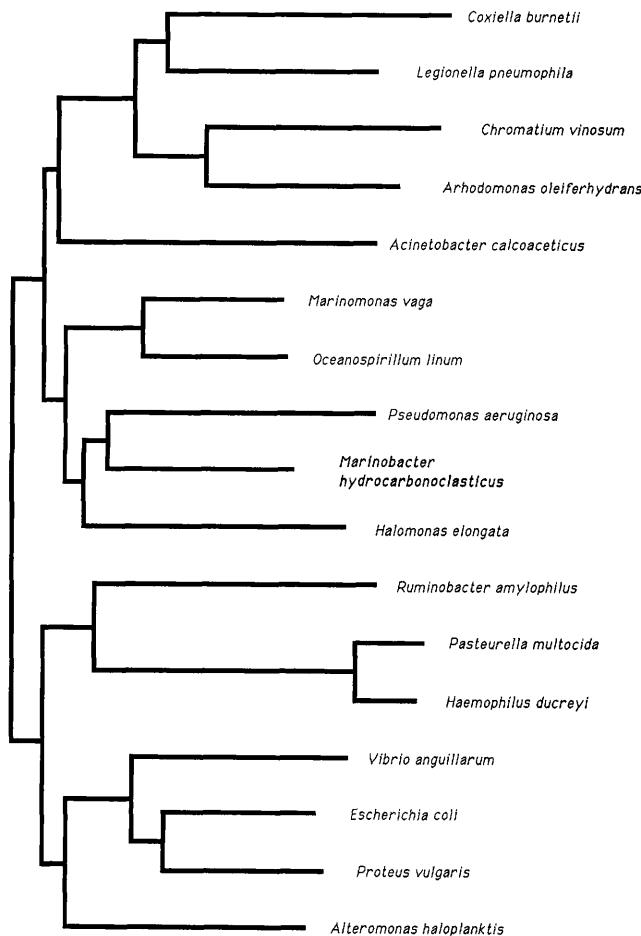


FIG. 6. Position of *M. hydrocarbonoclasticus* SP.17 (=ATCC 49840) within the gamma subgroup of phylum *Proteobacteria*. The figure was obtained by using a neighbor-joining method and the D2 domain. Strain SP.17 always clustered close to a number of bacteria, including *M. vaga*, *O. linum*, *P. aeruginosa*, and *H. elongata*.

Analysis of the 16S rRNA sequence results in compelling information about the taxonomic position of our strain. A nonambiguous affiliation of this bacterium is with the *Proteobacteria*, gamma group. The closest relatives for which a 16S rRNA sequence is presently known are *M. vaga*, *P. aeruginosa*, *O. linum*, and *H. elongata*, the last two being seemingly the most closely related, but it is not possible to determine a significant closest relationship to any single bacterium. This analysis does not preclude the possibility that further sequencing of 16S rRNAs of other bacteria may demonstrate a close relationship to a bacterium already well characterized phenotypically. We have tried, however, to avoid this possibility by sequencing the 16S rRNA of three bacteria having morphological and biochemical properties which indicated a possible relationship. Since this sequencing revealed a significant distance between SP.17 and the other species, we suggest that SP.17 be assigned at least temporarily to a new species in a new genus, under the epithet *Marinobacter hydrocarbonoclasticus* gen. nov., sp. nov., without excluding the possibility of a revision in regard to new data.

**Description of type strain.** The cells of the type strain of *Marinobacter hydrocarbonoclasticus* (Ma.ri'no.bac.ter. L.

TABLE 2. Dissimilarity between 16S rRNA sequences used in Fig. 6

| Organism                                  | % Dissimilarity <sup>a</sup>      |                                |                |                                   |                                    |                               |                                 |                    |                                   |                               |                                      |                                     |                |                 |                                |                                    |   |
|---|-----------------------------------|--------------------------------|----------------|-----------------------------------|------------------------------------|-------------------------------|---------------------------------|--------------------|-----------------------------------|-------------------------------|--------------------------------------|-------------------------------------|----------------|-----------------|--------------------------------|------------------------------------|---|
|   | <i>R. amylo-</i><br><i>philus</i> | <i>P. vul-</i><br><i>garis</i> | <i>E. coli</i> | <i>V. anguil-</i><br><i>larum</i> | <i>A. halo-</i><br><i>planktis</i> | <i>H. du-</i><br><i>cryei</i> | <i>P. multi-</i><br><i>cida</i> | <i>C. burnetii</i> | <i>L. pneumo-</i><br><i>phila</i> | <i>C. vin-</i><br><i>osum</i> | <i>A. oleifer-</i><br><i>hydrans</i> | <i>A. calco-</i><br><i>aceticus</i> | <i>M. vaga</i> | <i>O. linum</i> | <i>H. elon-</i><br><i>gata</i> | <i>P. aerugino-</i><br><i>sana</i> | <i>M. hydro-</i><br><i>carbon-</i><br><i>oclasticus</i> |
| <i>Proteus vulgaris</i>                   | 7.8                               | 3.5                            | 4.8            | 5.4                               | 5.4                                | 6.5                           | 6.5                             | 6.8                | 6.8                               | 6.8                           | 7.7                                  | 5.6                                 | 5.7            | 4.8             | 7.0                            |                                    |   |
| <i>Escherichia coli</i>                   | 6.0                               | 4.3                            | 5.9            | 6.2                               | 6.0                                | 5.6                           | 5.9                             | 7.6                | 7.6                               | 7.6                           | 8.1                                  | 8.5                                 | 8.1            | 8.5             | 7.1                            | 6.3                                |   |
| <i>Vibrio anguillarum</i>                 | 8.3                               | 9.2                            | 9.0            | 8.4                               | 7.9                                | 8.1                           | 8.1                             | 8.8                | 8.8                               | 8.5                           | 6.9                                  | 6.9                                 | 6.9            | 5.8             | 6.6                            | 3.1                                |   |
| <i>Alteromonas haloplanktis</i>           | 7.0                               | 7.9                            | 8.2            | 8.0                               | 9.9                                | 8.8                           | 8.8                             | 8.4                | 7.9                               | 8.5                           | 8.8                                  | 8.8                                 | 8.1            | 6.8             | 7.2                            | 5.4                                |   |
| <i>Haemophilus ducreyi</i>                | 6.9                               | 6.0                            | 5.6            | 6.8                               | 6.4                                | 5.9                           | 5.9                             | 7.6                | 7.6                               | 7.6                           | 7.9                                  | 7.9                                 | 7.8            | 7.4             | 7.3                            | 5.6                                |   |
| <i>Pasteurella multocida</i>              | 6.8                               | 6.4                            | 9.4            | 9.5                               | 9.8                                | 8.9                           | 8.9                             | 7.2                | 8.9                               | 8.9                           | 8.8                                  | 8.8                                 | 8.1            | 6.8             | 8.5                            | 6.0                                |   |
| <i>Coxiella burnetii</i>                  | 8.2                               | 7.9                            | 8.1            | 7.9                               | 8.0                                | 7.9                           | 7.9                             | 8.5                | 8.5                               | 8.5                           | 8.8                                  | 8.8                                 | 8.1            | 6.2             | 7.6                            | 7.1                                |   |
| <i>Legionella pneumophila</i>             | 6.9                               | 9.2                            | 8.2            | 8.0                               | 9.9                                | 8.8                           | 8.8                             | 8.4                | 8.4                               | 8.4                           | 8.8                                  | 8.8                                 | 8.1            | 8.1             | 6.7                            | 6.6                                |   |
| <i>Chromatium vinosum</i>                 | 7.9                               | 7.9                            | 8.2            | 8.2                               | 8.2                                | 8.2                           | 8.2                             | 8.4                | 8.4                               | 8.4                           | 7.2                                  | 7.0                                 | 8.0            | 6.1             | 7.2                            | 6.7                                |   |
| <i>Arthromonas oleiferyhydrans</i>        | 7.9                               | 7.9                            | 8.2            | 8.2                               | 8.2                                | 8.2                           | 8.2                             | 8.4                | 8.4                               | 8.4                           | 6.4                                  | 6.4                                 | 7.8            | 7.4             | 7.2                            | 6.7                                |   |
| <i>Acinetobacter calcoaceticus</i>        | 7.6                               | 8.1                            | 7.9            | 7.9                               | 8.0                                | 7.9                           | 7.9                             | 7.2                | 7.2                               | 7.2                           | 8.0                                  | 8.0                                 | 9.3            | 7.8             | 7.4                            | 7.1                                |   |
| <i>Marinomonas vaga</i>                   | 7.3                               | 6.2                            | 6.9            | 6.6                               | 6.0                                | 6.0                           | 6.0                             | 7.2                | 7.0                               | 7.0                           | 6.4                                  | 6.4                                 | 7.0            | 6.9             | 7.2                            | 6.1                                |   |
| <i>Oceanospirillum linum</i>              | 7.0                               | 5.3                            | 5.7            | 5.4                               | 6.1                                | 6.1                           | 6.1                             | 7.2                | 7.0                               | 7.0                           | 8.8                                  | 8.8                                 | 7.8            | 7.4             | 7.2                            | 6.7                                |   |
| <i>Halomonas elongata</i>                 | 8.5                               | 6.4                            | 7.5            | 7.5                               | 6.9                                | 6.9                           | 6.9                             | 7.5                | 7.5                               | 7.5                           | 9.3                                  | 9.3                                 | 9.3            | 7.8             | 7.4                            | 7.3                                |   |
| <i>Pseudomonas aeruginosa</i>             | 7.3                               | 8.2                            | 7.0            | 8.0                               | 8.0                                | 8.0                           | 8.0                             | 7.0                | 7.0                               | 7.0                           | 8.4                                  | 8.4                                 | 7.0            | 6.0             | 5.2                            | 5.3                                |   |
| <i>Marinobacter hydrocarbonoclasticus</i> | 7.1                               | 7.8                            | 7.5            | 8.0                               | 7.2                                | 8.6                           | 8.6                             | 7.2                | 8.0                               | 8.0                           | 7.5                                  | 7.5                                 | 7.0            | 6.9             | 7.2                            | 6.1                                |   |

<sup>a</sup> Percentages were calculated by using conserved domains of the molecule (referred to as D2 in Materials and Methods). Ambiguous or undetermined positions were ignored.

adj. *marinus*, of the sea; M. L. n. *bacter*, masc. equivalent of Gr. neut. n. *bacterion*, rod or staff; M. L. masc. n. *Marinobacter*, rod of the sea) (*hy'dro.car.bo.no.clas'ti.cus* M. L. part. adj. hydrocarbonoclastic, hydrocarbon dismantling) are rod shaped (2 to 3  $\mu\text{m}$  long and 0.3 to 0.6  $\mu\text{m}$  wide in logarithmic growth phase) and harbor numerous surface blebs when grown on eicosane in mineral medium. Cells are gram negative, nonsporeforming, and motile by means of a single unsheathed polar flagellum in media containing 0.2 to 1 M NaCl. They are unflagellated in media with a lower or higher NaCl concentration. Colonies on agar media are white when young and pinky beige after 48 h of incubation.

The cells are able to grow at temperatures ranging from 10 to 45°C (mesotrophic), with optimal growth at 32°C. They exhibit extreme halotolerance and can grow in NaCl concentrations ranging from 0.08 to 3.5 M. They have an absolute requirement for sodium ion. Aerobic, with a nonfermentative metabolism, they can grow anaerobically with nitrate or on succinate, citrate, or acetate but not on glucose. They are able to denitrify, with N<sub>2</sub> production. They exhibit oxidase, cytochrome oxidase, catalase, tweenase, and lecithinase activities. They grow on acetate, butyrate, caproate, succinate, fumarate, adipate, DL-lactate, and citrate as sole carbon sources but not on carbohydrates and amino acids (except L-proline and L-glutamate). They degrade a large variety of aliphatic or aromatic hydrocarbons and produce a nondialyzable bioemulsifier when grown on hydrocarbons.

SP.17 cells are resistant to novobiocin, tetracycline, oleandomycin, staphylomycin, and vibriostatic agent 0/129 and are susceptible to penicillin G, kanamycin, streptomycin, chloramphenicol, erythromycin, cephaloridine, gentamicin, and nalidixic acid.

The guanine-plus-cytosine content of the DNA, determined from measurements of thermal denaturation temperatures, is 52.7 mol%. According to the 16S rRNA sequence, the bacterium belongs to the gamma-type *Proteobacteria* and is part of a distinct monophyletic group including *O. linum*, *M. vaga*, *H. elongata*, *P. aeruginosa*, and *A. calco-aceticus*. Strain SP.17 was isolated from Mediterranean seawater (Gulf of Fos) near a petroleum refinery outlet. The type strain has been deposited with the American Type Culture Collection as strain ATCC 49840.

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