Characterization of a New Marine Sedimentary Bacterium as *Flavobacterium oceanosedimentum* sp. nov.

C. E. CARTY AND C. D. LITCHFIELD

Department of Microbiology and Center for Coastal and Environmental Studies: Marine Sciences, Rutgers-The State University, New Brunswick, New Jersey 08903

Bacterium J217 was isolated from approximately 100 cm below the sediment-water interface of the Irish Sea. It is a small, gram-negative, nonmotile rod with a guanine-plus-cytosine content of 67.5 mol%. J217 is an obligate aerobe that is catalase positive and cytochrome oxidase positive. It is nonfermentative, does not hydrolyze complex molecules, and is resistant to seven antibiotics but is susceptible to polymyxin B. Branched-chain amino acids are required for growth; flavine adenine dinucleotide or riboflavine 5'-phosphate and pyridoxal phosphate are necessary cofactors. The temperature, pH, and salinity are narrowly defined with optima at 23°C, pH 7.3, and 36‰ salinity, respectively. Sodium ions are required for growth and multiplication. Spectral analyses of the yellow, carotenoid pigment produced by J217 are similar to those associated with McMeekin type 2 bacteria. The data presented suggest that J217 be classified as *Flavobacterium oceanosedimentum* sp. nov.

Pigmented, gram-negative rods constitute a large proportion of the aerobic, heterotrophic bacteria isolated from marine sediments (6-9). Taxonomic studies of this group have been hindered not only by the overlapping definitions of the genera *Flavobacterium* and *Cytophaga*, but also by the relative difficulty of maintaining pigmented bacteria in culture collections (2, 12, 14). In previous papers, the isolation (9, 10) and some growth characteristics (7, 8) of the pigmented, marine, sedimentary bacterium J217 were described. This paper is a thorough report of the morphological, cultural, and biochemical properties of strain J217. Based upon these characteristics, it is proposed that the strain J217 be designated as *Flavobacterium oceanosedimentum* sp. nov.

(Portions of this report were presented at the annual meeting of the American Society for Microbiology, New Orleans, La., 8 to 13 May 1977 [abstr. N86, p. 243]. This work is taken from a Ph.D. thesis to be submitted by C. E. Carty.)

MATERIALS AND METHODS

Initial isolation, purification procedures, and tentative identification techniques have been previously described (9). Although other amino acid auxotrophs were isolated, this is the only strain obtained with these unique characteristics (9).

Culture maintenance. The culture is maintained on two media. The first is an enriched seawater medium containing 0.1% proteose peptone no. 3, 0.1% yeast extract, 0.05% sodium glycerophosphate, and 4% Rila sea salts (Utility Chemical Co., Paterson, N.J.). The second medium (four-salts medium) has the nu-

trient composition listed above, but the artificial seawater has been replaced with a defined salts solution consisting of 0.4 M NaCl, 0.01 M CaCl₂, 0.01 M KCl, and 0.028 M MgSO₄·7H₂O. The pH of both media is 7.3 ± 0.1.

Gram reaction and motility. Broth cultures were Gram-stained at various stages of the growth curve; the crystal violet and iodine solutions were made in artificial seawater, but otherwise the standard technique was used (15). Motility was examined by the hanging-drop technique.

Colonial morphology. The colonial morphology was described after 13 days at 23°C on seawater medium agar.

Gliding motility. The ability to glide on a solid surface was examined. A cover slip was placed over a colony formed on the surface of a seawater medium agar plate, and the edge of the colony was examined by oil immersion light microscopy.

Electron microscopy. A mid-log-phase broth culture was negatively stained with a 2% solution of phosphotungstic acid (pH 7). An electron micrograph was prepared at a magnification of ×24,000 (Siemens Elmiskop 1A, Cherry Hill, N.J.).

Antibiotic susceptibility. The antibiotic susceptibility of J217 to eight different antibiotics was tested on surface-seeded seawater medium agar plates, using sensitivity disks obtained from Difco except for those disks containing 10 µg of O/129, which were prepared in our laboratory. The antibiotic concentrations tested were: ampicillin (10 U); erythromycin (15 µg); chloramphenicol (30 µg); penicillin (10 U); streptomycin (30 µg); tetracycline (30 µg); and polymyxin B (300 U).

DNA base composition. The moles percent guanine plus cytosine was determined by the spectrophotometric method of Ulitzur (16).

Growth ranges. Nephelometer flasks (250 ml) containing 80 ml of seawater medium were inoculated with 0.01 ml of a washed, early-log-phase broth culture

and incubated for 48 days. The broth was modified as needed for each test. Flasks were checked on a daily basis; growth was monitored spectrophotometrically at 620 nm with a Bausch and Lomb Spectronic 20 colorimeter.

Temperature range. Cultures were incubated at 0, 4, 15, 20, 23, 25, 30, 37, 44.5, and 55°C.

pH range. Flasks of seawater medium broth were buffered at pH 4, 5, 6, and 7, using 0.01 M phosphate buffer; at pH 8 and 9, 0.02 M borate buffer was used. Cultures were incubated at 23°C.

Salinity range. Flasks of modified four-salts medium broth were used in these studies. The defined salts solution described above was omitted; sufficient NaCl was added to give final concentrations of 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10% (wt/vol). Cultures were incubated at 23°C and shaken at 125 rpm in a New Brunswick Scientific Psychrotherm.

Ion requirements. Modified four-salts broth media were used in this test; one or all of the constituents of the defined salts solution was omitted from the medium; sodium glycerophosphate was replaced by glycerol. Flasks were inoculated with 1 ml of an early-log-phase four-salts medium broth culture which had been washed twice with 0.01 M *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer. Cultures were incubated at 4, 15, 23, and 30°C for 45 days.

Carbohydrate reactions. The ability of isolate J217 to produce acid from sugars was determined by using seawater-containing media and the method of Hugh and Leifson (4). Cultures were incubated at 23°C and examined on a daily basis for 21 days. The carbohydrates tested were: glycerol, arabinose, ribose, xylose, fructose, galactose, glucose, mannose, lactose, and sucrose.

FIG. 1. Negatively stained preparation of *F. oceanosedimentum*. Mid-log-phase cells were stained with 2% (wt/vol) phosphotungstic acid. Bar indicates 1 μ m.

Biochemical tests. All media contained 36‰ seawater and were inoculated with 0.1 ml of a washed, early-log-phase seawater medium broth culture. The temperature of incubation was 23°C; the incubation time varied with the test. Oxidase activity was determined by the method of Kovacs (5). Arginine dihydrolase activity was detected by the technique of Møller (13). Other biochemical reactions were monitored by standard methods (15) and/or by rapid diagnostic tests (Patho-Tec Rapid I-D System, Warner-Lambert Co., Morris Plains, N.J.; and API 20E, Analytab Products, Ayerst Laboratories, Plainview, N.Y.).

Cell yield. Final cell yield was determined by measuring the dry weight of cell suspensions filtered onto 0.22- μ m filters; the dry weight was corrected for residual salt.

Nitrogen requirements. The basal medium used in these tests contained 0.1% yeast extract and 0.05% sodium glycerophosphate in the above-described four-salts medium. To 80 ml of basal medium was added 1 ml of a filter-sterilized nitrogen solution; the final nitrogen concentration was 0.1 or 0.01% (wt/vol).

Vitamin requirements. The basal medium used in these studies contained 0.1% proteose peptone no. 3–0.05% sodium glycerophosphate in 4% artificial seawater. To 9 ml of basal medium was added 1 ml of a filter-sterilized cofactor solution; the final concentration of vitamins was approximately 0.001% (wt/vol).

Pigment extraction. Fermentor-grown seawater medium broth cells harvested at late-log phase were used in these studies. Pigments were extracted by the method of Vaisberg and Schiff (17).

Spectral analysis of pigments. The ultraviolet-visible absorption spectrum of the pigment extract was determined in chloroform and hexane on a recording Varian 634S scanning double-beam spectrophotometer. The molar extinction coefficient was determined by the method of Davies (1).

Chemicals. Throughout these studies analytical grade or better quality reagents were used.

RESULTS

Morphology. Small, smooth, concave colonies formed on solid media have entire edges and are bright yellow, opaque, and approximately 1 mm in diameter. Microscopic examination of the edges did not reveal the existence of gliding motility.

The bacterium is a small (0.9 by 1.5 μ m), gram-negative rod which is nonmotile. A negatively stained whole-cell preparation of the culture is shown in Fig. 1. The photomicrograph confirms the observations made by phase-contrast microscopy regarding the small size of J217 as well as its lack of flagella and/or gliding strands.

Cultural and physiological characteristics. This isolate is an obligate aerobe that is catalase positive and cytochrome oxidase positive. The latter was tested by both standard methods (15) and API 20E series. It is Kovacs oxidase negative, and it does not contain nitrate reductase, urease, arginine dihydrolase, lysine

decarboxylase, ornithine decarboxylase, or phenylalanine deaminase.

J217 does not hydrolyze casein, gelatin, starch, or Tween 80. It fails to produce indole and H_2S and cannot utilize citrate. It is also negative for the Voges-Proskauer and methyl red tests.

In Hugh-Leifson marine media it will grow but does not produce a color change when glycerol, arabinose, ribose, xylose, fructose, galactose, glucose, mannose, lactose, or sucrose is present. In addition, there is growth but no color change in phenol red broth supplemented with glycerol, ribose, glucose, or lactose.

J217 requires the branched-chain amino acids leucine, valine and/or isoleucine for growth, and NH_4Cl cannot substitute even when glycerol, pyruvate, glycerophosphate, arabinose, or glucose is present.

This strain is resistant to seven antibiotics tested: ampicillin, erythromycin, chloramphenicol, O/129, penicillin, streptomycin, and tetracycline. It is susceptible to polymyxin B.

Deoxyribonucleic acid base composition. The guanine-plus-cytosine content of J217 is 67.5 mol% when determined by the method of Ulitzur (16). Values for control cultures, *Escherichia coli*, *Staphylococcus aureus*, and *Micro-*

FIG. 2. Effect of temperature on the growth of *F. oceanosedimentum* in seawater broth.

FIG. 3. Effect of pH on the growth of *F. oceanosedimentum* in seawater broth.

FIG. 4. Effect of salinity on the growth of *F. oceanosedimentum* in seawater broth.

coccus lysodeikticus were within 0.7 mol% of reported values.

Growth characteristics. The effect of temperature on the growth of isolate J217 is shown in Fig. 2. The minimum growth temperature is 5°C, whereas 35°C is the maximum temperature permitting growth. The optimum growth temperature is 23°C.

Figure 3 shows the effect of pH on final cell yield. The pH optimum of J217 is pH 7.3; this bacterium grows over the range bounded by pH 5.5 and pH 8.1.

The salinity optimum of J217 is 36‰ S (Fig. 4). The minimum and maximum salinities tolerated by this organism are 10‰ S and 80‰ S, respectively. This salinity requirement is not osmotic, as there is a specific requirement for sodium ions (Table 1).

TABLE 1. Cation requirements for the growth of *Flavobacterium oceanosedimentum*

Test cation	Concn (mmol)	Cell yield (mg/10 ml)
Four salts		1.9
Ca ²⁺ omitted	10	1.9
Mg ²⁺ omitted	28	1.8
K ⁺ omitted	10	0.1
Na ⁺ omitted	400	0
Na ⁺ omitted, Li ⁺ added	400	0
Control ^a		2.1

^a Control consisted of Rila sea salts prepared according to manufacturer's instructions.

TABLE 2. Specific nitrogen requirements for the growth of *Flavobacterium oceanosedimentum*^a

Nitrogen source	Nitrogen concn (mg/10 ml)	Final cell yield [mg (dry wt)/10 ml]
Ammonium ions	100	0
	10	0
Nitrate	100	0
	10	0
Casein	100	1.2
	10	0.8
Proteose peptone no. 3	100	2.0
	10	1.1
L-Isoleucine	100	1.2
	10	0.6
L-Leucine	100	1.4
	10	0.5
L-Valine	100	1.1
	10	0.3
L-Isoleucine + L-leucine	50	1.8
	50	
Other L-amino acids	100	0
	10	0

^a Basal medium: 0.1% yeast extract, 0.05% sodium glycerophosphate, and 4% Rila sea salts.

The general nitrogen requirements of J217 are best satisfied by the addition of proteose peptone no. 3 to the basal medium (Table 2). However, the specific nitrogen requirement is fulfilled by the addition of the L-isomers of branched-chain amino acids to the medium. Other amino acids, ammonium ions, and nitrate cannot be used as sole sources of nitrogen (Table 2). Other compounds may stimulate the growth of this bacterium (Table 3), but only pyridoxal phosphate and either flavine adenine dinucleotide or riboflavine 5'-phosphate are essential co-factors.

Pigmentation pattern. The absorption spectra in both hexane and chloroform are shown in Fig. 5. From the crude extract three maxima are detected which occur in a pattern

TABLE 3. Vitamin requirements for the optimal growth of *Flavobacterium oceanosedimentum*^a

Source ^b	Concn (μ g/10 ml)	Cell yield [mg (dry wt)/10 ml]
Biotin	10	0
Folic acid	10	0
FAD	10	0
FMN	10	0
Pyridoxal phosphate	10	0
FAD + pyridoxal phosphate ...	5	
	5	1.70
FMN + pyridoxal phosphate ...	5	
	5	1.66
Yeast extract	10	1.90

^a Basal medium: 0.1% proteose peptone no. 3, 0.05% sodium glycerophosphate, and 4% Rila sea salts.

^b FAD, Flavine adenine dinucleotide; FMN, riboflavine 5'-phosphate.

that is characteristic of carotenoids. In hexane, the peak occurs at 445 nm and has distinct inflections at 430 and 470 nm. In chloroform, the curve is shifted 5 nm toward the red end of the light spectrum. The molar extinction coefficient of the crude pigment extract is 2,440.

DISCUSSION

A large proportion of the bacteria isolated from marine and estuarine sediments are chromogenic, gram-negative rods (2, 3, 6, 20-22). Their identification and classification have been hindered not only by their cultural fastidiousness but also by the confused state of their taxonomy. Indeed, the applications of numerical taxonomy and nucleotide base analysis to the genera *Flavobacterium* and *Cytophaga* have emphasized the random and arbitrary manner by which these organisms have been classified (3, 12, 18, 19). However, assignation of strain J217 to the genus *Flavobacterium* is justified on the basis of the following characteristics: lack of hydrolytic enzymes, failure to ferment sugars, oxidative metabolism, and lack of motility. J217 differs from other members of the genus *Flavobacterium* group II (19) in only two aspects. The first is the nutritional fastidiousness of this organism in requiring L-form branched-chain amino acids or complex protein for growth; this aspect is under investigation. The other major difference concerns the absorption spectrum of the pigment. The crude extract is more similar to those reported for group I species (12). Final chemical identification of the pigment via chromatography, mass spectroscopy, and derivati-

FIG. 5. Absorbance spectra of the crude pigment extract of *F. oceanosedimentum* in chloroform (—) or in hexane (---).

zation will be reported separately. It is sufficient to reiterate that the taxonomy of flavobacteria is sufficiently arbitrary that neither of these two differences should be considered as crucial. Accordingly, we propose that organisms currently referred to as isolate J217 be classified as *Flavobacterium oceanosedimentum*, (o·ce·an·o·sed·i·mén·tum. L. adj. *ocean*o from the sea; L. adj. *sedimentum* from the sediment; M.L. fem. adj. *oceanosedimentum* intended to mean from marine sediments). The type strain is American Type Culture Collection (ATCC) strain 31317.

The marine nature of *F. oceanosedimentum* is attested to by its absolute requirement for sodium ions for growth (11). The specific nature of this requirement is presently unknown, but neither potassium ion nor lithium ion can substitute for the sodium ion requirement. Moreover, the pH optimum (pH 7.3) is near that usually encountered in marine sediments. Despite the slightly elevated (for marine bacteria) optimum temperature of 23°C, its temperature limits would allow the organism to survive and grow in marine sediments. Further characterization of the lipids and pigments of *F. oceanosedimentum* as well as its unusual nitrogen requirements will be reported separately.

ACKNOWLEDGMENTS

We thank John Quinn for his skillful technical assistance.

This work was supported by U.S. Environmental Protection Agency grant R804865 and by NOAA-MESA grant 04-6-022-44020. C. E. Carty acknowledges support from National Science Foundation grant no. SMI-75-22042 to SMI-77-22042.

REPRINT REQUESTS

Address reprint requests to: Dr. Carol D. Litchfield, Center for Coastal and Environmental Studies, Marine Sciences, Rutgers—The State University, New Brunswick, NJ 08903.

LITERATURE CITED

1. Davies, B. H. 1965. Analysis of carotenoid pigments, p. 489-532. In T. W. Goodwin (ed.), *Chemistry and biochemistry of plant pigments*. Academic Press Inc., London.
2. Floodgate, G. D., and P. R. Hayes. 1961. The preservation of marine bacteria. *J. Appl. Bacteriol.* **24**:87-93.
3. Floodgate, G. D., and P. R. Hayes. 1963. The Adansonian taxonomy of some yellow pigmented marine bacteria. *J. Gen. Microbiol.* **30**:237-244.
4. Hugh, R., and E. Leifson. 1953. The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various gram-negative bacteria. *J. Bacteriol.* **66**:24-26.
5. Kovacs, N. 1956. Identification of *Pseudomonas pyocyanea* by the oxidase reaction. *Nature (London)* **178**:703.
6. Liston, J. 1968. Distribution, taxonomy and function of heterotrophic bacteria on the sea floor. *Bull. Misaki Mar. Biol. Inst. Kyoto Univ.* **12**:97-104.
7. Litchfield, C. D. 1973. Interactions of amino acids and marine bacteria, p. 145-168. In L. H. Stevenson and R. R. Colwell (ed.), *Belle W. Baruch Library in Marine Science, vol. I, Estuarine microbial ecology*. University of South Carolina Press, Columbia, S.C.
8. Litchfield, C. D. 1974. Nonequivalence of proteins from marine and conventional sources for the cultivation of marine bacteria, p. 354-362. In R. Y. Morita, and R. R. Colwell (ed.), *Effect of the ocean environment on microbial activities*. University Park Press, Baltimore.
9. Litchfield, C. D., and G. D. Floodgate. 1975. Biochemistry and microbiology of some Irish Sea sediments. II. Bacteriological analyses. *Mar. Biol.* **30**:97-103.
10. Litchfield, C. D., A. L. S. Munro, L. C. Massie, and G. D. Floodgate. 1974. Biochemistry and microbiology of some Irish Sea sediments. I. Amino acid analyses. *Mar. Biol.* **26**:249-260.
11. MacLeod, R. A. 1965. The question of the existence of specific marine bacteria. *Bacteriol. Rev.* **29**:9-23.
12. McMeekin, T. A., J. T. Patterson, and J. G. Murray. 1971. An initial approach to the taxonomy of some Gram negative yellow pigmented rods. *J. Appl. Bacteriol.* **34**:699-716.
13. Møller, V. 1955. Simplified tests for some amino acid decarboxylases and for the arginine dihydrolase system. *Acta Pathol. Microbiol. Scand.* **336**:158-172.
14. Prince, H. N., E. S. Beck, R. C. Cleverdon, and W. L. Kulp. 1954. The flavobacteria. I. Nutritional requirements. *J. Bacteriol.* **68**:326-328.
15. Society of American Bacteriologists. 1957. *Manual of Microbiological methods*. McGraw-Hill, Toronto.
16. Ulitzur, S. 1972. Rapid determination of DNA base composition by ultraviolet spectroscopy. *Biochim. Biophys. Acta* **272**:1-11.
17. Vaisberg, A. J., and J. A. Schiff. 1976. Inhibition of carotenoid biosynthesis by the herbicide SAN 9789 (4-chloro-5-(methylamine)-2-(α,α,α -trifluoro-m-tolyl)-3-(2(H)pyridazinone). *Plant Physiol.* **57**:260-269.
18. Weeks, O. B. 1969. Problems concerning the relationship of cytophages and flavobacteria. *J. Appl. Bacteriol.* **32**:13-18.
19. Weeks, O. B. 1974. Genus *Flavobacterium*, p. 357-364. In R. E. Buchanan and N. E. Gibbons (ed.), *Bergey's manual of determinative bacteriology*, 8th ed. The Williams and Wilkins Co., Baltimore.
20. ZoBell, C. E. 1941. Studies on marine bacteria. I. The cultural requirements of heterotrophic aerobes. *J. Mar. Res.* **4**:42-75.
21. ZoBell, C. E. 1946. *Marine microbiology*. Chronica Botanica, Waltham, Mass.
22. ZoBell, C. E., and H. C. Upham. 1944. A list of marine bacteria including descriptions of sixty new species. *Bull. Scripps Inst. Oceanogr.* **5**:239-292.