Alteromonas citrea, a New Gram-Negative, Yellow-Pigmented Species from Seawater

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Three strains of marine bacteria that produce a lemon-yellow, non-carotenoid pigment and a polyanionic antibiotic substance have been investigated from phenotypic and genetic standpoints. Their phenotypic characters, together with the low guanine-plus-cytosine contents of their deoxyribonucleic acids, place these strains in the genus *Alteromonas* Baumann et al. The main features of these strains are sufficiently different from those of the alteromonads previously described to justify placing them in a new species, for which the name *Alteromonas citrea* is proposed. The type strain is strain no. 10 (=NCMB 1889).

Previous studies on antibiotic-producing bacteria isolated from seawater led us to describe a group of gram-negative rods (5) whose major genotypic and phenotypic characters were similar to those of members of the genus *Alteromonas* Baumann et al. (2). From this group, two new species have previously been described: *Alteromonas luteoviolaceus* (3) and *Alteromonas rubra* (4).

The study reported here was carried out to determine the taxonomic position of three strains of yellow-pigmented alteromonads chosen from several similar strains isolated from Mediterranean waters off Nice. Like *A. rubra* and *A. luteoviolaceus*, these bacteria produce in seawater media a polyanionic antibiotic which probably acts on bacterial respiration.

MATERIALS AND METHODS

Bacterial strains. The three strains reported here were isolated from the surface of water from the Mediterranean Sea near Nice. The isolation medium used was marine agar (MA) (Difco). The strains were selected for study on the basis of two characteristics: their lemon-yellow pigmentation and their antibiotic activity against a test bacterium (*Staphylococcus epidermidis* Institut Pasteur Collection strain no. 53124). They were placed in the collection of the Centre d'Etudes et de Recherches de Biologie et d'Océanographie Médicale under the numbers 2, 4, and 10. Strain 10 was deposited in the National Collection of Marine Bacteria (Torry Research Station, Aberdeen, Scotland) as NCMB 1889.

Phenotypic analysis. The methods and the compositions of the media used in this study were previously described (3, 4). Only additional techniques or significant modifications of the previously described media and methods referred to above will be presented below. Unless stated otherwise, all cultures were grown on MA or in marine broth (Difco) and were incubated at 23°C.

Analysis of pigments. The fractionation of chloroform-methanol (1:1) extracts of cells by high-pressure liquid chromatography (Waters Associates liquid chromatograph, type ALC-GPC 244) was made by elution with acetonitrile-tetrahydrofuran-water (1:1:2) in a MicroBondapak C18 packed column (4-mm inner diameter by 30 cm long) with detection at 436 nm.

Utilization of organic compounds. Besides the 34 organic compounds used in a previously described study of alteromonads (4), the following additional substrates were employed: D-ribose, deoxyribose, D-glucosamine, D-galactosamine, D-glucuronate, L-glutamate, L-ascorbate, oxalate, tributyrin, L-proline, DL-asparagine, L-lysine, L-ornithine, L-arginine, DL-histidine, DL-threonine, L-cysteine, glycyl-glycine, DL-alanine, DL-phenylalanine, DL-tryptophane, DL-leucine, urea, inulin, *m*-hydroxybenzoate, and *p*-hydroxybenzoate. Utilization of water-soluble products was determined as described by Baumann et al. (1, 2), using BM Casamino Acids medium, and utilization of water-insoluble compounds was determined by the method of Véron (10) on BM Casamino Acids plus agar (Difco) (0.5%, wt/vol). Growth-inhibiting substrates (citrate, glycerol, D-tartrate, *o*-hydroxybenzoate, *m*-hydroxybenzoate, *p*-hydroxybenzoate, and D-glucosamine) were tested at different final concentrations (1, 0.1, and 0.01%, wt/vol).

Production of TMA from TMAO. The production of trimethylamine (TMA) from trimethylamine oxide (TMAO) was determined in the medium of Wood and Baird (NaCl, 2 g; MgSO₄, 0.1 g; K₂HPO₄, 0.1 g; D-glucose, 0.25 g; peptone [Difco], 0.5 g; and distilled water, 100 ml) with 0.1% TMAO (wt/vol) added. TMA was detected by use of the technique of Laycock and Regier (7) modified by using 20% KOH instead of saturated K₂CO₃.

Production of antibiotics. Antibiotic production was studied by scraping off 1 g of wet cells from the surface of MA at the end of the logarithmic phase of growth (24 h), suspending it in 20 ml of half-strength seawater with 10 mM ethylenediaminetetraacetic acid, centrifuging (5,000 rpm, 5 min, 4°C), and precipitating the supernatant with cold ethanol (1:1). The precipitate was then dissolved in distilled water, dialyzed

against distilled water for 48 h, and concentrated under vacuum (30°C) to 5 ml. The antibiotic activity of this extract was evidenced on tryptic soy agar (Difco) seeded with *S. epidermidis* strain Institut Pasteur no. 53124. The electrophoretic behavior of antibiotics was studied on Cellogel strips (Chemetron, Milan, Italy) in Veronal buffer (pH 9.2) under 200 V for 45 min. The development of strips was made on solid medium (tryptic soy agar) seeded with cells of *S. epidermidis* strain Institut Pasteur 53124.

RESULTS

Morphology. All of the cells were straight rods with rounded ends and were gram negative when stained in the exponential phase of growth (24 h). They usually occurred singly, but some filaments or chains were evident in old cultures (6 to 10 days). After 24 h, the cells appeared to be from 0.7 to 1.5 μm in width and from 1.5 to 4.0 μm in length. All cells were motile, except for the filamentous forms, by means of a single polar flagellum (Fig. 1). The motility strongly decreased after 3 to 4 days in either liquid or solid media. None of the strains produced endospores or capsules.

Cultural characteristics. None of the three strains grew in the absence of oxygen, and thus they are strict aerobes.

On MA, colonies were very small (0.5 mm in diameter), bright, and whitish after 24 h at 23°C. Yellow pigmentation appeared in the center of the colonies after 48 h. After 4 days, the colonies were circular (4 to 5 mm in diameter), regular, convex, bright, smooth, and uniformly lemon-yellow; no diffusible pigment was produced in the medium. When starch (5%, wt/vol) was added to the medium, large (6 to 7 mm in diameter), brownish-yellow colonies were produced which were surrounded by an opalescent ring showing starch hydrolysis.

On blood MA, the three strains produced large (6 to 7 mm in diameter), bright, mucoid, whitish colonies; the medium turned black around the colonies after 2 or 3 days. Each colony was surrounded by a ring of complete hemolysis after 5 days.

On tryptic soy agar and on nutrient agar (Difco), both prepared with distilled water and 2% NaCl (wt/vol), only strain 4 grew, and it produced very small, pale-yellow colonies. On the same media prepared with seawater, the three strains produced larger colonies (3 to 4 mm in diameter). A strong increase in lemon-yellow pigment production occurred in the colonies grown on these media to which ferric phosphate (0.1%, wt/vol) had been added. None of the strains produced diffusible pigments.

On King media A and B (6), all of the strains produced large pale-yellow colonies; the medium

FIG. 1. Electron micrograph of strain 10 (=NCMB 1889). Bar represents 1 μm .

around the colonies turned slightly brownish-yellow.

After 4 days in marine broth at 23°C, growth occurred in the top centimeter only, and a thin, more or less discontinuous, lemon-yellow pellicle, a colored ring on the glass, and a very slight, pale-yellow sediment were produced.

In a nutrient gelatin (Difco) stab containing 2% NaCl (wt/vol), infundibuliform liquefaction took place rapidly (48 h) around the stab; the hydrolysate was a limpid colorless fluid.

Temperature, pH, salinity, and sodium requirements. Strain 4 was able to grow at temperatures from 10 to 40°C and tolerated pH values from 6 to 12. Strains 2 and 10 were less tolerant: they could grow only at temperatures from 10 to 30°C and at pH values from 6 to 10. None of the strains grew in artificial seawater medium with a salinity lower than 0.8‰; growth was normal with salinities from 1 to 11‰ and slow at 11.5‰. All of the strains required Na^+ for growth (Fig. 2). Despite some differences in their sensitivities to that ion, all of the strains were inhibited by Na^+ concentrations lower than 0.5 M and higher than 1 M. Strains 2 and 10 were less tolerant to variations in the Na^+ concentration, their optimum growth occurring with concentrations of 0.5 to 0.6 M Na^+ .

FIG. 2. Sodium requirements of strains 4 and 10 (=NCMB 1889) studied by means of a recording biophotometer; incubation in stirred medium at 23°C. Symbols: ●, maximum absorbance of cultures; ○, duration of lag phase.

Antibiotic production. Antibiotic production was greatest in MA and generally in media prepared with seawater. In MA, this production was constant whatever the pH; it was maximum at low temperatures for strains 2 and 10, whereas for strain 4 it was more active at high temperatures (37 to 40°C). In MA, strains 2 and 10 produced two polyanionic antibiotics with different electrostatic charges; strain 4 produced only one active product (Fig. 3). In all cases, antibiotic synthesis was very low on blood MA: the lack of activity might be due to the presence of catalase in blood (4). It was also lower in nutrient agar, tryptic soy agar, and King medium A.

Pigment production. All three strains produced a non-carotenoid, lemon-yellow pigment in seawater media. Pigment production was enhanced by the addition of starch and ferric phosphate to the medium. The absorption spectra of chloroform-methanol extracts of the pigments produced by the three strains were markedly similar: each showed a maximum absorption peak at 438 nm irrespective of the pH of the extract (Fig. 4). These pigments were, in fact, composed of several fractions which could be separated chromatographically. High-pressure liquid chromatographs (Fig. 5) showed the pigments produced by the three strains to be highly heterogeneous: strains 2 and 10 produced a closely related mixture of yellow-pigmented fractions, whereas the main fraction of pigments synthesized by strain 4 was markedly different.

Range of organic compounds utilized. Of the sixty organic substrates tested, only D-glucose, trehalose, D-mannose, D-fructose, starch, and tributyrin were utilized as sole sources of carbon by the three strains. In addition, strains

FIG. 3. Electrophoresis of antibiotics isolated from cells of strains 2, 4, and 10. The Cellologel strips were placed on the surface of a solid medium seeded with *Staphylococcus epidermidis* strain Institut Pasteur no. 53124 and removed, for photography, after 18 h at 37°C.

2 and 10 were able to utilize asparagine and proline, and strain 4 used oxalate, histidine, and threonine. None of the strains was able to use

intermediary metabolites of the tricarboxylic acid cycle or *ortho*-, *meta*-, and *para*-hydroxybenzoate.

Polyhydroxybutyrate accumulation. The three strains were unable to utilize DL- β -hydroxybutyrate, and they did not accumulate its polymer.

FIG. 4. Absorption spectra of crude chloroform-methanol (1:1) extracts of the pigments produced by strains 2 (----), 4 (—), and 10 (····).

FIG. 5. Fractionation of the chloroform-methanol (1:1) extracts of the lemon-yellow marine strains by high-pressure liquid chromatography.

Production of TMA from TMAO. None of the strains produced TMA from TMAO.

Enzyme production. Each of the three strains possessed the following constitutive enzymes: catalase, oxidase, cytochrome oxidase, amylases, deoxyribonuclease, phospholipase C, Tween esterase, tributyratase lipase, tryptophan deaminase, phenylalanine deaminase, acid phosphatase, alkaline phosphatase, gelatinases (trypsin and chymotrypsin-like enzymes), aminopeptidases (leucine, valine, cystine), and phosphoamidase. The catalase activity was weak and irregular. Only strains 2 and 10 showed a marked β -glucosaminidase activity, and only strain 4 possessed a chitinase. None of the three strains produced a nitrate reductase, a β -galactosidase, a tryptophanase, a urease, an arginine dihydrolase, a β -glucuronidase, an α -mannosidase, an α -fucosidase, α - and β -glucosidases, and lysine and ornithine decarboxylases.

Susceptibility to inhibitors: (i) antibiotics. All strains were susceptible to erythromycin, spiramycin, oleandomycin, vancomycin, chloramphenicol, thiophenicol, rifamycin, and neomycin. They were slightly inhibited by streptomycin, kanamycin, polymyxin, and colimycin and were totally resistant to tetracycline and chlortetracycline. Marked differences between the strains appeared in their susceptibilities to some other antibiotics. Thus, bacitracin was active only against strain 10, and penicillin and novobiocin were active only against strains 2 and 10.

(ii) Vibriostatic pteridin (0/129). All of the strains were resistant to pteridin (0/129), possi-

bly because the test was carried out in a saline medium (4).

(iii) **Respiratory effectors.** All strains were strongly inhibited by KCN, acriflavine, and 8-hydroxyquinoline and were resistant to lysozyme and urethane.

Miscellaneous reactions. None of the strains produced H₂S, gave a positive methyl-red or Voges-Proskauer reaction, reduced methylene blue, or hydrolyzed esculin. All of the strains rapidly hydrolyzed casein.

DNA base composition. The guanine-plus-cytosine (G+C) contents of the deoxyribonucleic acids (DNAs) of strains 2, 4, and 10 were 42.2, 44.7, and 41.5 mol%, respectively.

DISCUSSION

On the basis of their phenotypic characters, the three strains fall into group IV of Shewan of the genus *Pseudomonas* (9). However, the strains can be assigned to the genus *Altero-*

monas Baumann et al. (2) since the G+C contents of their DNAs are lower than those of pseudomonads. This genetic character also differentiates these strains from the several lemon-yellow bacteria studied by McMeekin et al. (8).

The Na⁺ requirements of strains 2 and 10, and to a lesser degree those of strain 4, permit these strains to be included in Lee's group (or phenon) C (J. V. Lee, Ph.D. thesis, Aberdeen University, Scotland, 1973): all required Na⁺ for growth, no growth having been observed with low concentrations of this ion (0.005 M Na⁺). Nevertheless, strains 2 and 10 are more sensitive to high concentrations of Na⁺ than are the alteromonads of group C, and they require a higher Na⁺ concentration for optimum growth (0.6 M instead of 0.3 M Na⁺).

The strains investigated in this study have a number of characters in common and a few differences with the alteromonads previously described (Table 1). Their G+C values are close

TABLE 1. Characters which distinguish the three lemon-yellow marine strains from the previously named species of *Alteromonas*

Character	<i>A. commu-</i> <i>nis</i> ^a [33] ^f	<i>A. vaga</i> ^a [17]	<i>A. macleo-</i> <i>dii</i> ^a [21]	<i>A. halo-</i> <i>planktis</i> ^a [17]	<i>A. luteovio-</i> <i>laceus</i> ^b [16]	<i>A. rubra</i> ^c [3]	Yellow-pig- mented ma- rine strains [3]
Straight rods	—	+	+	+	+	+-	+
Oxidase	+	—	+	+	+	+	+
Catalase	ND ^f	ND	+	ND	—	(+)	(+)
Amylases	—	—	(19) ^g	(1)	(15)	+	+
Gelatinases	—	—	(20)	+	+	+	+
Chitinase	—	—	—	(14)	—	—	(1)
Lipase	—	—	+	+	+	+	+
Acid from:							
D-Glucose	+	+	+	+	(9)	+	(+)
D-Mannose	(29)	+	—	(15)	—	+	+
Maltose	(26)	(6)	+	+	+	—	—
Trehalose	(6)	(3)	(19)	(13)	+	+	+
Cellobiose	—	(14)	+	—	—	—	—
Succinate	+	+	—	+	ND	—	—
α-Ketoglutarate	+	+	—	—	ND	—	—
Erythritol	—	+	—	—	ND	—	—
Glycerol	+	+	+	—	—	—	—
Ring cleavage	+	+	—	—	ND	—	—
<i>m</i> -OH benzoate	+	+	—	—	ND	—	—
Violacein	—	—	—	—	(14)	—	—
Prodigiosin	—	—	—	—	—	+	—
Yellow pigment	—	—	—	—	(5)	—	+
Production of antibiot- ics	ND	ND	—	ND	+	+	+
Growth at 40°C	+	—	(15)	—	—	—	(1)
Mol% G+C	47	48	46	43	42	47	42

^a Data from Baumann et al. (2).

^b Data from Gauthier (3).

^c Data from Gauthier (4).

^d Figures in brackets are numbers of strains examined.

^e Symbols: +, all strains positive; —, all strains negative; +—, variable; (+), slight reaction or growth.

^f ND, Not determined.

^g Figures in parentheses are numbers of positive strains.

to those of *Alteromonas haloplanktis* and *Alteromonas luteoviolaceus*. However, the yellow-pigmented strains have a marked amylase activity, are unable to use maltose and succinate as sole sources of carbon, and synthesize a macromolecular autotoxic substance and several yellow pigments; such characteristics are not present in *A. haloplanktis*. Furthermore, they are significantly different from *A. luteoviolaceus*: thus, they do not produce violacein, they do possess a catalase activity, they are able to use mannose but not maltose, and their yellow pigments are quite different from that of *A. luteoviolaceus*.

The morphological, physiological, and biochemical similarities between strains 2 and 10 allow these strains to be grouped together in a single species that is sufficiently different from other alteromonads so as to be considered a new species, for which the name *Alteromonas citrea* (cit're.a. L. adj. *citreus* lemon yellow) is proposed. Strain 10 (=NCMB 1889) is designated the type strain of this species.

In spite of some physiological differences, strain 4 appears to be closely related to the type strain. At the present time, however, it cannot be assigned to a unique and separate species because only a few differential features have been found to distinguish it from the type strain.

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