Taxonomic Position and Seasonal Variations in Marine Neritic Environment of Some Gram-negative Antibiotic-producing Bacteria

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SUMMARY

Six marine bacteria which synthesize macromolecular antibiotics were isolated from neritic waters on the French Mediterranean coast, and their frequency recorded over two successive years.

They appeared in relatively large numbers during the period August to December, and can be identified as marine pseudomonads; however, the low guanine-cytosine ratio of their DNA, lack of catalase and specific self-inhibition are not compatible with the characteristics of the genus *Pseudomonas*. Two produced violacein, usually synthesized by bacteria belonging to the genus *Chromobacterium*. Their taxonomic position is discussed.

INTRODUCTION

Many marine bacteria release antibacterial substances into culture media. These substances inhibit various terrestrial and in particular Gram-positive bacteria. Marine bacteria are widespread; they have been found in the Atlantic Ocean (Rosenfeld & ZoBell, 1947; Grein & Meyers, 1958; Buck, Meyers & Kamp, 1962; Buck, Ahearn, Roth & Meyers, 1963; Buck & Meyers, 1965, 1966; Burkholder, Pfister & Leitz, 1966; Doggett, 1968), the Indian Ocean (Baam, Gandhi & Freitas, 1966; Lebedeva & Markianovic, 1971), the Mediterranean Sea (Gauthier, 1969; Lebedeva & Markianovic, 1971), the Red Sea and the Gulf of Aden (Lebedeva & Markianovic, 1971) and the Baltic Sea (Bonde, 1968). They have been isolated from most marine biotopes, including surface or deep waters, immersed substrates, surface of seaweeds, and fine or rough sediments, and have been identified as species of *Bacillus*, *Micrococcus*, *Pseudomonas*, *Vibrio*, *Flavobacterium*, *Alcaligenes*, *Xanthomonas* and *Achromobacter*.

Bacteria isolated from reasonably unpolluted Mediterranean coastal waters near Nice are characterized by the macromolecular and polysaccharide nature of their antibacterial substance and by the mechanism of their inhibition of terrestrial bacteria, mainly Gram-positive species but also a few Gram-negative ones such as *Klebsiella* spp. and *Escherichia* spp. (Gauthier, 1970). Seasonal variations in the numbers of such bacteria within neritic heterotrophic bacterial populations over two successive years (1969–1970) are reported, together with their morphological, physiological and biochemical characters.

METHODS

Bacteria. The six strains studied were Gram-negative heterotrophic rods, which periodically occurred in relatively large numbers in the water or at the surface of immersed substrates in the littoral area near Nice. The area is rocky and calcareous, and covered with an algal population of *Cystoseira*. Samples were taken at 10 m from the shore at a depth of 6 m.

A single strain of each type studied was deposited in the C.E.R.B.O.M. collection (strains Nos. 6, 10, 18, 56, 114 and 123), later being acceded to the National Collection of Marine Bacteria (Torry Research Station, Aberdeen) as NCMB strains 1943, 1889, 1890, 1892, 1942 and 1941, respectively.

Media and enumeration. Bacterial counts were performed fortnightly for two years, using either filtration (Millipore filters, 0.45 μ m pore size) or direct spreading of samples on agar plates of ZoBell's medium 2216 E (Bactopeptone 5 g, yeast extract 1 g, FePO_4 0.1 g, aged seawater 800 ml, distilled water 200 ml, pH 7.6). On this medium, they showed characteristic pigmentations which proved useful for rapid identification: NCMB1943, pinkish beige with reddish-brown diffusible pigment; NCMB1889, lemon yellow; NCMB1890, bright red, turning carmine in old cultures; NCMB1892, orange, soon becoming greenish-brown; NCMB1942, light violet; NCMB1941, dark violet, almost black.

Their antibiotic activity against *Staphylococcus aureus* (Institut Pasteur, strain 209p) was tested by the double-layer technique used for studying bacteriocin production (Gratia & Fredericq, 1946). A small volume of each sample (0.1 to 1 ml) of an appropriate dilution was spread on the surface of the medium and incubated for 6 days at 20 °C. After being killed with chloroform (30 min), the colonies were covered with a thin layer (1 mm) of Trypticase soy agar sown with *S. aureus* (209p). After 18 h at 37 °C, the antibiotic-producing colonies showed a clear zone of inhibition. The violet colonies were counted separately. The counts of each of these pigmented types were then compared with the total number of heterotrophs in the sample.

Microscopy. Flagella were demonstrated by a modification of Rhodes' (1958) method. It was impossible to wash the bacteria before preparing the smears and especially before mordant action because of lysis, so rinsing was done very rapidly with 10 % seawater, best results being obtained by doubling the concentration of the tannic acid solution.

Lysis also made it difficult to prepare specimens for electron microscopy. The bacteria, grown for 24 h on solid medium, were suspended in 5 % (w/v) formalin containing 3 % (w/v) NaCl. After rinsing 2 or 3 times in saline formalin and centrifuging (2800 g, 15 min, 10 °C), the pellet was resuspended in 5 % (w/v) ammonium acetate, the bacteria shadowed with Au/Pd, and observed in a Siemens Elmiskop type 1 electron microscope.

General physiological and biochemical properties. The identification scheme for Gram-negative rods proposed by Shewan, Hobbs & Hodgkiss (1960) and Hendrie, Hodgkiss & Shewan (1964) was used. The violet pigments of strains NCMB1941 and NCMB1942 were extracted, purified and identified as described by Strong (1944).

Since the bacteria grew only on a medium of high ion content, sensitivity to O/129 was done on ZoBell's medium 2216 E. Caution has to be exercised in interpreting negative results because this medium could inhibit the activity of O/129 (Merkel, 1972).

Sodium requirements. These were studied with a biophotometer (Bonet-Maury, 425 nm), in 'mock seawater' medium (Casitone 0.4 %, yeast extract 0.2 %, NaCl 1.75 %, KCl 0.1 %, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ 0.4 %, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.1 %; tris 0.05 %, distilled water 100 ml, pH 7.6 with N-HCl) in the presence of increasing concentrations of NaCl (0.001, 0.05, 0.1, 0.2, 0.4, 0.6, 0.8 and 1 M). Inoculation was carried out in biophotometer cells, with 0.05 ml of a thick

Fig. 1. Flagella stain of strain NCMB1890, as described in text. This strain, like the five other types, is monotrichous and polar.

Fig. 2. Electron micrograph of strain NCMB1890 showing typical pseudocapsule formation.

bacterial suspension in a medium containing 0.1 M-Na^+ . The duration of the lag phase and the maximum absorbance attained by each strain in each medium were measured.

Lytic phenomena. By using the technique of Chabbert (1963) the number of surviving bacteria was determined in a suspension of each strain in artificial saline solution (KCl , NaCl , $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ and $\text{CaCl}_2 \cdot 4\text{H}_2\text{O}$), successively deprived of sodium, magnesium and calcium, or of all three elements simultaneously. The absorbance of each suspension was measured 1 min, 1 h and 18 h after mixing, seawater, distilled water and sucrose (1 M) being used as references.

Base composition of DNA. The guanine-cytosine (GC) ratio of the DNA was determined by thermal denaturation (Marmur & Doty, 1962) after extraction and purification of the DNA by the technique of Marmur (1961). It was not possible to purify DNA from strain NCMB1892.

Quantitative variations during the year. The numbers of each of the six bacterial types in the water were obtained by the standard method of plating and counting, which underestimates the true count in the sample. However, since the total heterotrophic count was obtained in the same way, the proportion of antibiotic-producing strains in the whole population is probably accurate.

RESULTS

Taxonomic features

All microscopic observations (Figs. 1 and 2) showed the bacteria to be straight Gram-negative rods, of between 1.5×1.4 and $4 \times 1.4 \mu\text{m}$, motile by means of a single polar flagellum. The electron-dense pericellular zone suggested the presence of a loose pseudocapsule, but this could have been an artefact.

All were oxidase- and cytochrome oxidase-positive, but catalase-negative, insensitive to the vibriostat O/129 and to penicillin, and unable to ferment glucose in Leifson's (1963) medium. None produced fluorescent pigment in King's (1954) media A and B, or showed any growth at temperatures higher than 30°C (except for strain NCMB1890). All were strongly proteolytic and readily hydrolysed starch, but were unable to reduce nitrates. The pigment

Fig. 3

Fig. 3. Absorption spectrum of the pigment produced by NCMB1941 (-----) and NCMB1942 (- · - · - · -) in ethanol, and of pure violacein (—) (taken from Sneath, 1956a).

Fig. 4

Fig. 4. Sodium requirements of the strain NCMB1941 studied by means of a recording biophotometer (with incubation in stirred medium at 25 °C). The other five strains gave similar results, except NCMB1890 which was less inhibited by Na^+ concentrations higher than 0.6 M. ●, Maximum absorbance of cultures; ○, duration of lag phase.

produced by strains NCMB1941 and NCMB1942 appeared identical with violacein (Fig. 3), usually synthesized by *Chromobacterium* spp.; it gave colour reactions specific to this pigment: emerald green in sulphuric acid, bright blue in acetic acid, and green turning yellow in 10 % KOH.

None of the bacteria grew in media containing less than 0.2 M- Na^+ , the optimum for growth being 0.4 to 0.6 M- Na^+ , i.e. approximately the same as in seawater. They were inhibited in higher concentrations (Fig. 4). Sodium also seems essential for maintaining the integrity of their walls, since they lysed in a few seconds in a sodium-free solution even in the presence of high concentrations of other ions (K^+ , Ca^{2+} , Mg^{2+}) (Table 1). The absence of divalent cations did not cause any cellular deterioration. Moreover, a saline solution with only Na^+ and K^+ salts could prevent much of the lysis, whereas Mg^{2+} or Ca^{2+} salts were ineffective. Sucrose did not prevent lysis.

These results agree with those of other workers who have suggested that the need for Na^+ in metabolism and in the maintenance of the wall is one of the main characteristics of the 'true' marine bacteria (Scholes & Shewan, 1964; McLeod, 1965; DeVoe & Oginsky, 1969a, b).

The GC ratios of the DNA of all the bacteria were relatively low: 46.9, 41.5, 47.1, 42.2 and 42.0 % for NCMB1943, NCMB1889, NCMB1890, NCMB1942 and NCMB1941, respectively.

Table 1. *Effect of Na⁺, Ca²⁺ and Mg²⁺ on cell lysis*

Solutions*	Ions present	Percentage of bacteria surviving																	
		NCMB1943			NCMB1889			NCMB1890			NCMB1892			NCMB1942			NCMB1941		
		1 min	1 h	18 h	1 min	1 h	18 h	1 min	1 h	18 h	1 min	1 h	18 h	1 min	1 h	18 h	1 min	1 h	18 h
Seawater	All	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Distilled water	None	0.001	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sucrose (1 M)	None	0.1	0	0	1	0	0	0.1	0	0	10	0	0	0	0	0	0	0	0
Solution 1	K ⁺ Na ⁺ Ca ²⁺ Mg ²⁺	100	100	100	100	100	100	100	100	50	100	100	100	100	100	100	100	100	100
Solution 2	K ⁺	0.001	0	0	0	0	0	0.001	0	0	0	0	0	0	0	0	0	0	0
Solution 3	K ⁺ Ca ²⁺	0.01	0	0	0	0	0	0.1	0	0	0	0	0	0	0	0	0	0	0
Solution 4	K ⁺ Ca ²⁺ Mg ²⁺	1.0	0	0	1.0	0	0	100	0.5	0	1.0	0	0	0	0	0	0	0	0
Solution 5	K ⁺ Na ⁺	100	100	100	100	100	10	100	100	50	100	50	100	50	100	10	100	50	10
Solution 6	K ⁺ Na ⁺ Ca ²⁺	100	100	100	100	100	100	100	100	100	100	100	100	100	100	0	100	100	100
Solution 7	K ⁺ Mg ²⁺	1.0	0	0	0.5	0	0	10	0.5	0	0.5	0	0	0	0	0	0	0	0
Solution 8	K ⁺ Na ⁺ Mg ²⁺	100	100	100	100	100	100	100	100	100	50	50	100	50	100	50	100	50	50

* Solution 1 contained (g/l distilled water): NaCl, 17.5; MgCl₂.6H₂O, 4.0; CaCl₂.4H₂O, 1.0; KCl, 1.0. Components were omitted to give solutions 2 to 8 as indicated.

Fig. 5. Seasonal variations in the counts of the six bacterial types, over two successive annual cycles (1969-70). (a) ●, NCMB1892; ○, NCMB1943. (b) ●, NCMB1889; ■, NCMB1941-1942; ▲, NCMB1890.

Quantitative variations during the year.

In the marine biotope studied, the six bacterial types mainly appeared during the second half of the year, with maximum densities in September and October for strains similar to NCMB1943, NCMB1890, NCMB1892, NCMB1941 and NCMB1942, and from November to January for strain NCMB1889 (Fig. 5).

DISCUSSION

The six bacteria isolated can be placed in *Pseudomonas* group IV of Shewan *et al.* (1960). However, their low % GC relative to recognized *Pseudomonas* species (60 to 70% GC), the absence of catalase and their sodium requirements remove them from this genus. They are similar to the marine pseudomonas studied by Lee (1973), which show a strong similarity to *Alteromonas* (Baumann, Baumann, Mandel & Allen, 1972).

Assuming that they are 'marine pseudomonads', these bacteria are distinguished by their strong antibacterial properties. Gauthier (1970), using NCMB1890 and some violet types, showed that the characteristics of the antibiosis appear to be closely related to the structure of the bacteria. The substances responsible for the activity are polysaccharides, probably bound to the outside layers of the wall, and could constitute the pseudocapsule seen in electron micrographs. These antibacterial substances inhibit the bacteria producing them, during their lag and stationary phases, so giving them a short life in the usual culture media. They must be subcultured every 4 to 5 days, whatever the medium. The antagonistic activity is partly or totally neutralized by catalase either added to the medium or present in the bacteria used for antibiosis tests (Gauthier, 1972). This is similar to the 'catalase effect' described by Sneath (1956*a*) for species of *Chromobacterium*, for which no mechanism of antibiosis has been described. The absence of catalase and their property of self inhibition

probably involves major modifications in the respiratory metabolism of the bacteria studied here. They may differ in their structure and functions from the true pseudomonads, and may thus be a physiologically isolated group.

Two strains, NCMB1941 and NCMB1942, produced violacein, which was studied by Leifson (1956) and Sneath (1956*a*) in the genus *Chromobacterium*, and by Hamilton & Austin (1967) in *Chromobacterium marinum*. *Chromobacterium marinum* also has a very short life on solid media and its reported properties are similar to those of NCMB1941 and NCMB1942. It was included in the genus *Chromobacterium* because it produced violacein, showed a 'catalase effect' and had a mixed flagellation (Sneath, 1956*b*), properties regarded as specific to the genus. Both NCMB1941 and NCMB1942 contain violacein and show the 'catalase effect'. However, their flagellation is always monotrichous and polar. The presence of the same pigment in two different bacteria does not necessarily imply a close phylogenetic relationship between them. Starr (1958) showed that the blue pigments of *Corynebacterium insidiosum* and *Pseudomonas indigofera* are identical. In addition, the % GC of the genus *Chromobacterium* (65 to 72 %; De Ley & Van Muylen, 1963) is much higher than that of NCMB1941 and NCMB1942, and such a difference is sufficient to exclude them from the genus. Thus the 'catalase effect' can no longer be regarded as specific to the genus *Chromobacterium*.

The growing interest in marine microbiology is due in part to the existence of species with properties which enable them to survive and grow under the often unfavourable conditions in the marine environment. Some physiological properties have been used to try to define the marine nature of strains isolated from the sea, but the problem of whether there exist specifically marine bacteria remains unresolved. In our opinion the best definition of marine bacteria is that they are the only bacteria able to multiply in the sea and play an active part in the life-cycle of oceans. Such a definition fits the bacteria described here. They require seawater for growth and survival, appear regularly in each annual cycle during the autumn phytoplanktonic blooms rich in diatoms, and are present for a good part of the winter. At times they form more than 5 % of the bacterial heterotrophs which can be cultured in the laboratory. They disappear when the phytoplanktonic population becomes composed of Dinoflagellates. The role of these bacteria in the sea is not known but their special inhibitory properties may play some part in determining the relationships within the microbial ecology of the sea.

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