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Production of a Pyrrole Antibiotic by a Marine Bacterium[1]

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ABSTRACT

BURKHOLDER, PAUL R. (Lamont Geological Observatory, Palisades, N.Y.), ROBERT M. PFISTER, AND FREDERICK H. LEITZ. Production of a pyrrole antibiotic by a marine bacterium. Appl. Microbiol. 14:649–653. 1966.—Evidence is presented for the isolation and identification of bacteria able to synthesize an unusual antibiotic containing five bromine atoms per molecule. The identification and taxonomic position of these bacteria was made by use of a computer in conjunction with traditional methods. These microorganisms and closely related strains have been isolated on various occasions from tropical water in the vicinity of Puerto Rico. One bacterium, a pseudomonad, has been given the name *Pseudomonas bromoutilis* because of its distinctive capability. The antibiotic has been extracted, purified, and obtained in crystal form, and its structure has been determined. Although clinical tests of its properties were not encouraging, it may be of significant value and interest from an ecological standpoint.

This report concerns the properties of a marine bacterium which produces a new antibiotic substance, and the laboratory methods employed in the production and isolation of the compound. The determination and proof of its structure [2,3,4-tribromo-5(1′ hydroxy, 2′,4′-dibromo phenyl)pyrrole] will be described by Lovell (1).

MATERIALS AND METHODS

The bacterium was isolated upon several occasions from *Thalassia* located near La Parguera, Puerto Rico. The isolation medium had the following ingredients added to 1 liter of seawater: N-Z Case, 2.0 g; Soytone, 1.0 g; yeast extract, 1.0 g; and Difco agar, 20 g. About 1.0 g of turtle grass was shaken in 99 ml of sterile seawater, and 1 ml of the suspension was then plated into pour plates of the nutrient seawater agar. The plates were incubated overnight at room temperature in the laboratory of Marine Biology at La Parguera. Colonies were observed as yellow-tan in color with a tendency to cause some darkening of the agar. The isolates were grown and maintained on the same nutrient seawater agar. Two isolates, obtained at different times and designated strains 287 and 396, were selected for special studies.

The media and tests were prepared and used according to descriptions given previously (2). The observations included standard properties of morphology, cultural characteristics, substrate utilization, antibiotic relationships, carbon sources, and the response to pteridine 0/129.

RESULTS

Results obtained with cultures of strains 287 and 396 are presented in Tables 1 and 2.

The morphological properties of the cells of these two isolates were compared in a Philips 200 electron microscope. Cells were fixed in osmic acid, washed in water, and mounted on electron microscope grids. The electron micrographs are shown in Fig. 1.

Taxonomic relationships. The taxonomic relationships of these bacteria were studied by use of Adansonian principles and the clustering techniques described by Sokal and Sneath (4), the computer methods described by Pfister and Burkholder (3), and the more traditional techniques described in *Bergey's Manual.* The organisms were compared by use of a high-speed digital computer with a population of various marine pseudomonads isolated from Antarctic waters, members of the family *Achromobacteraceae,* a representative of the genus *Flavobacterium,* and a gelatin-liquefying methyl red-positive member of the *Pseudomonadaceae* isolated from tropical water. Single linkage cluster analysis of this group of microorganisms indicated that the bacteria (396 and 287) were distinct from these other marine species and formed an entirely sep-

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TABLE 1. *Taxonomic data obtained for two isolates of an antibiotic-producing marine bacterium*

Property	Strain 287	Strain 396
Single rods	+	+
Pleomorphic	+	+
Size length	0.6–1.2 μ	>1.2 μ
Size width	0.5 μ	>1.0 μ
End of cell	Rounded	Rounded
Motility	+	+ (weakly)
Flagellation	Unipolar	Unipolar (when flagellated)
Gram reaction	Negative	Negative
Colony size	>1.1 mm	>1.1 mm
Edge	Entire	Entire
Colony appearance	Raised convex	Raised, flat
Agar stroke	Filiform	Filiform
Pigmentation	Translucent, brown	Translucent, brown
Diffusible pigment	Tan to brown	Tan
Relative growth	Rapid	Rapid
Turbidity in broth	Even	Even
Temperature for growth	20 to 35 C	20 to 35 C
Requirement for seawater	+	+
Growth in distilled water media	−	−
Produces antibiotic	+	+
Sensitive to bacitracin	No	No
Sensitive to penicillin	No	No
Sensitive to tetracyline	No	No
Sensitive to viomycin	No	No
Sensitive to novobiocin	Yes	Yes
Sensitive to oleandomycin	Yes	Yes
Agar digested	No	No
Starch digested	Yes	Yes
Gelatin liquified	Yes	Yes
Voges-Proskauer	Negative	Negative
Methyl red	Negative	Negative
Indole production	Negative	Negative
Citrate utilization	+	+
Urease	+	−
Catalase	−	−
NH_4NO_3 utilized	No	+
N-Z Case utilized	Yes	Yes
Nitrate reduced	No	No
H_2S formed	+	No
Tributyrin hydrolyzed	Yes	No
Triolein hydrolyzed	No	No
Pteridine O/129	No inhibition	No inhibition

arate cluster by themselves. This independent clustering, the lack of positive identification with standard techniques, and the accumulated descriptive and biochemical data (Tables 1 and 2) suggested to us that these bacteria should be placed in the genus *Pseudomonas*. Culture 396 has the strongest numerical relationship based upon per cent similarities (%S) when compared with other similar antibiotic-producing isolates from the same tropical area (e.g., 287). This is seen in Table 3 where %S was computed by comparing all the morphological and biochemical characteristics used to describe each bacterium with every other bacterium, resulting in the diagonal matrix shown (2). This comparison was made with six related organisms, each having a %S value with each of the other five. The average %S was calculated from these five values. In the case of strain 396, the average %S equals 79.0, the highest in this group of bacteria. These data indicate that this microorganism is most centrally located in the cluster and should be regarded as the type culture. Culture 396, because of its interesting new antibiotic substance containing 5 bromine atoms per molecule and its potential ecological significance, has been given the name *Pseudomonas bromoutilis*.

Production of antibiotic substance. In a preliminary screening survey of marine bacteria for antimicrobial activity, the isolates 287 and 396

TABLE 2. *Carbon sources for two isolates of an antibiotic-producing marine bacterium*

Carbon source	Strain 287		Strain 396	
	Aerobic	Anaerobic	Aerobic	Anaerobic
Glucose	+	−	Slight	−
Fructose	+	−	Slight	−
Mannose	+	−	Slight	−
Galactose	+	−	−	−
Xylose	−	−	−	−
Rhamnose	−	−	−	−
Arabinose	−	−	−	−
Lactose	−	−	−	−
Sucrose	+	−	+	+
Maltose	+	−	+	−
Trehalose	+	−	+	+
Melibiose	−	−	+	−
Raffinose	−	−	+	−
Dextrin	+	−	+	−
Inulin	−	−	−	−
Mannitol	−	−	−	−
Sorbitol	−	−	−	−
Dulcitol	−	−	−	−
Adonitol	−	−	−	−
Inositol	−	−	−	−
Levulose	−	−	−	−
Salacin	−	−	−	−
Glycerol	−	−	−	−

were observed to inhibit gram-positive laboratory strains of bacteria, *Candida albicans*, and many isolates of marine bacteria. The strains did not inhibit themselves, each other, or certain red antibiotic marine bacteria.

Preliminary attempts to grow strain 396 in liquid shake culture for production of the antibiotic were not entirely satisfactory, although various media, different speeds of shaking, and forced aeration were employed. Always the yields were less than could easily be obtained by growing the organism on the surface of agar plates. It was decided, therefore, to use agar in Pyrex baking dishes, for which special aluminum lids were fabricated. The formula of the medium was as follows: seawater, 1 liter; N-Z Case, 2 g; Soytone, 2 g; yeast extract, 1 g; dextrose, 2 g; vitamin B_{12}, 1 μg; and Difco agar, 20 g. Inoculum was grown in liquid medium without the agar. The seawater nutrient agar was autoclaved at a pressure of 15 psi for 20 min, and then 100 ml was dispensed into each of 50 heat-sterilized baking dishes. After the agar had cooled and solidified, 1 ml of a 24-hr actively growing suspension of the cells was streaked with a glass rod over the surface. Incubation was at 30 C for 3 days. The cells were scraped off the agar surface with a plastic windshield scraper and frozen at −15 C.

Isolation of the antibiotic compound was begun when approximately 50 g of the crude bacteria was available. The bacterial sludge was mixed with 5 volumes of methanol in a Waring Blendor for 5 min, and the viscous slurry was then centrifuged at 3,000 × *g* for 10 min. The supernatant liquid was decanted, and the remaining cellular sludge was returned to the blender; the procedure was repeated three times. The combined methanolic extracts were reduced in vacuo to yield a dark oily residue.

The residue was repeatedly stirred with ethyl ether until no further color was extracted. The ether was then removed, and the residue was taken up in chloroform. The chloroform solution was chromatographed on a silica gel (No. 3405; Baker Chemical Co.) column according to the usual procedure, with 100% chloroform used as the solvent. A series of fractions was assayed with *Bacillus subtilis* plates to determine the location of the antibiotic activity. The fractions which contained the antibiotic were combined and taken to dryness. This residue was dissolved in a minimal amount of chloroform and then cooled to −10 C. Long needles separated out of the cold solution, and these were then recrystallized from chloroform. The crystals gave a faint green color, and on heating began to decompose between 135 and 155 C. Upon heating to 200 C, only a black tar remained.

The crystals are very soluble in acetone, ethyl ether, and ethyl acetate, and are moderately soluble in chloroform and methanol. The compound is not soluble in water. Thin-layer chromatographic analysis of the purified material on silica gel G (according to Stahl) showed a single spot at R_F 0.50 with chloroform as the solvent and iodine vapor and concentrated sulfuric acid as the developing reagents. Bacteriological assay of the thin-layer chromatography plates showed only one zone of inhibition. Two other antibiotics originally present in the crude extracts (R_F = 0.0 and R_F = 0.55, with silica gel and chloroform) were shown to be absent in the crystalline material.

DISCUSSION

The biological properties of this pyrrole compound are of special interest in relation to its great activity against gram-positive bacteria. Strains of *Staphylococcus aureus*, *Diplococcus pneumoniae*, and *Streptococcus pyogenes* are inhibited completely at drug concentrations of about 0.0063 μg/ml in Trypticase Soy Broth (according to information provided by John

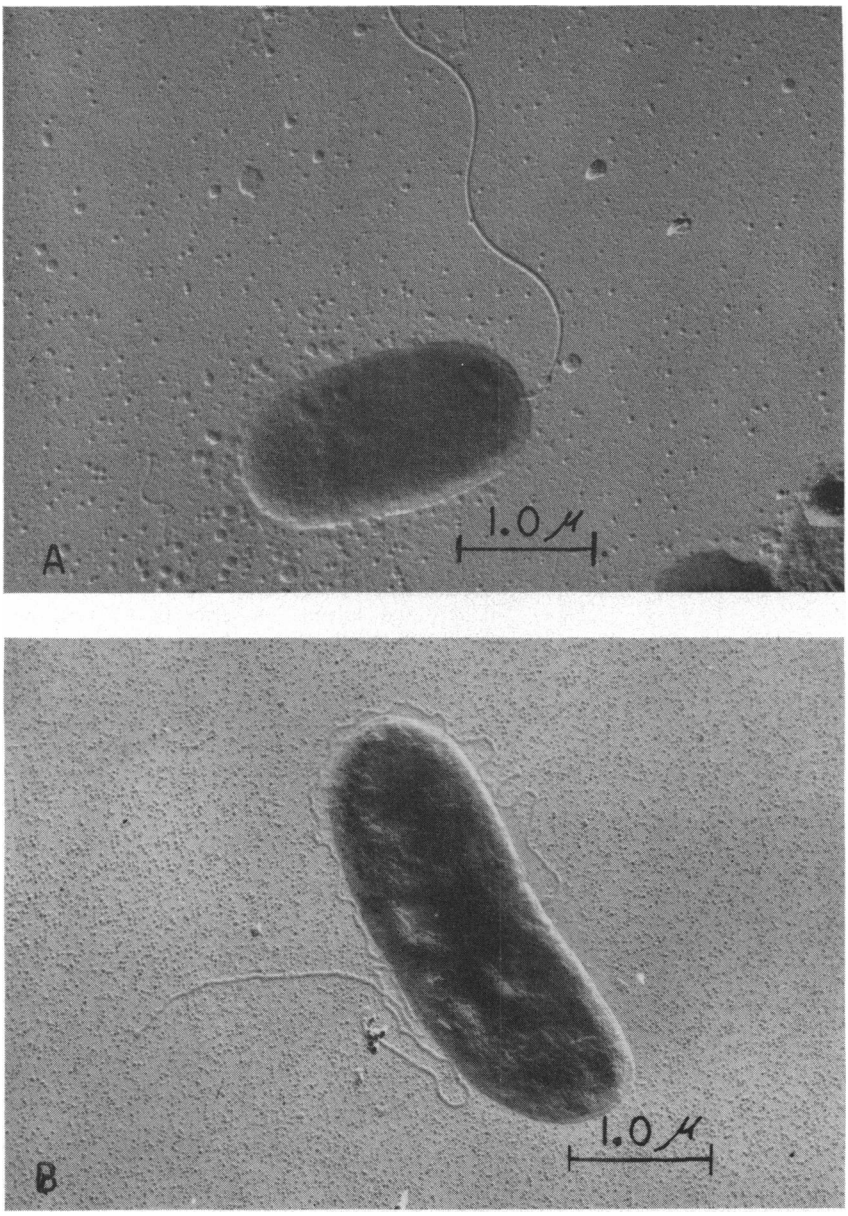

FIG. 1. *Upper bacterium (A) is a representative of culture 287. The lower picture (B) is a cell from culture 396.* × *18,000.*

TABLE 3. *Computed per cent similarity (%S) among cultures of a cluster*

Culture no.	Culture no.						Mean %S
	396	397	399	401	442	287	
396	100.0						79.0
397	75.6	100.0					78.2
399	77.4	81.9	100.0				78.2
401	74.7	77.4	79.2	100.0			75.1
442	81.9	84.6	79.2	74.7	100.0		78.5
287	85.5	72.0	73.8	69.3	72.9	100.0	74.6

Ehrlich, Parke, Davis & Co.). *Mycobacterium tuberculosis* in a synthetic medium was inhibited by 0.2 μg/ml. Gram-negative bacteria and *Candida albicans* were not affected at reasonable concentrations in broth or agar plate tests.

Toxicity of the antibiotic in mice was not ob-observed by intravenous injections of 25 mg/kg, but death resulted immediately upon injection of 50 mg/kg of a flocculent suspension in saline. No toxicity was obvious when 250 mg/kg was injected subcutaneously. Mouse protection tests with *Staphylococcus aureus*-infected animals showed negative results up to 200 mg/kg by subcutaneous administration. It is possible that insolubility and instability of the compound may have contributed to therapeutic failure in vivo.

The importance of antimicrobial substances for chemical ecology of the sea is obvious, in view of the inhibitory properties of the *Pseudomonas* for many bacteria isolated from the marine environment. The selective activities of the pyrrole compound and of other substances produced by marine organisms probably exert special controls in the complex population dynamics of marine microorganisms.

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