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海底堆積物より分離された多糖生産菌の分類学的研究

奥　谷　康　一

TAXONOMICAL STUDIES OF A POLYSACCHARIDE-PRODUCING BACTERIUM FROM MARINE SEDIMENT

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田辺湾の海底堆積物より海水培地を用いて分離された細菌 No. M-812 菌株は，蔗糖加寒天培地上の増殖で粘質多糖体を生産する．この菌株は海水培地に生育良好で，グラム染色陰性，胞子を形成せず，0.5×1.0～1.2μ の短桿状を示し，極鞭毛で運動する．カタラーゼ及びオキシダーゼ反応は陽性，グルコースから酸を産生するがガスの産生は見られない．この様な主な菌学的性状に加え，各種培地に対する生育及び生化学的性状から *Vibrio* 属と同定されるが，Bergey's manual（第8版）及び既報の海洋性 *Vibrio* 記載中には類似する菌種は認められない．

An extracellular polysaccharide-producing bacterium No. M-812 strain has been isolated from the marine sediment. The isolate was a Gram-negative, asporogenous rod, straight, 0.5×1.0–1.2 microns in size, motile with a single polar flagellum and grew well in sea water media. The catalase and oxidase tests were positive. Acid was produced from glucose, but no gas was formed.

From morphological, physiological and biochemical characteristics, the organism was classified into the genus *Vibrio*. However, it can be differenciated from all of five species described in the genus *Vibrio* according to the Bergey's manual.

Introduction

In the course of screening for extracellular polysaccharide-producing bacteria from marine environments, the present author isolated a strain from the marine sediment. The purpose of this study is to identify taxonomically this polysaccharide-producing bacterium named No. M-812 strain.

Materials and Methods

Isolation Procedures

A bacterial strain was isolated from the sediment sample collected at Kogaura in the Tanabe Bay, Wakayama. A loopful of the sediment sample was placed on sucrose-sea water (SSW) agar plate (Peptone 5 g, Yeast extract 1 g, Sucrose 30 g, Agar 15 g and Sea water 1 *l*) and carefully spread over the surface with a small sterile spreader made of glass rod. Inoculation was performed by twenty successive platings. After inoculation, all plates were incubated at 27°C for 4–5 days. The viscous colony grown on the medium was picked and streaked for isolation onto SSW agar plate. This isolate was purified by successive platings on SSW agar plates and was maintained on SSW agar slant. The bacterial culture was then subjected to study on morphological, cultural and biochemical characteristics.

Morphological Characteristics

A bacterial culture, grown on SSW agar medium for 24, 48 and 72 hr at 27°C was examined for motility, using

the hanging drop preparation. Motility was also observed by using SIM medium (Nissui Co.). Flagella arrangement was examined by Leifson's method for staining flagella[1]. Cell form was recoeded by the simple stain technique of Loeffler[1]. Gram-reaction was examined by the modified method of Hucker[1]. Spore formation was examined by staining a old (7–10 days) agar slant culture with methylene blue or by the method of Moeller's spore staining technique[1]. A surer test was also performed by emulsifying some of the old culture of a agar slant growth in 5 m*l* of the sterile 3% NaCl solution and heating it at 85°C for 10 min, afterward inoculating some of the heated material into SSW broth (Peptone 5 g, Yeast extract 1 g, Sucrose 30 g and Sea water 1 *l*) and incubating for several days. If the growth occur, it is practically certain that spores were present.

Cultural Characteristics

The growth features on SSW agar plate and SSW broth, MacConkey agar, CVT agar and PEA agar (Nissui Co.) were recorded. The presence of soluble, non soluble or fluorescent pigments was determined by examining cultures inoculated onto King A and B media (Nissui Co.) prepared with 3% NaCl solution.

The isolate was tested for its ability to grow under the reduced oxygen condition using a anaerobic-pyrogallol jar (Eiken Chemical Co.). For this test, the SSW agar plate was used.

Growth at 5, 10, 15, 20, 25, 30, 37 and 42°C, and the growth in 0–10% (1% each) NaCl were determined by using SSW broth and fresh water nutrient broth (Peptone 5 g, Yeast extract 1 g and Tap water 1 *l*), respectively.

Biochemical Characteristics

The isolate was subjected to test the biochemical characteristics. The sea water agar or sea water broth (Peptone 5 g Yeast extract 1 g, with or without Agar 15 g, and Sea water 1 *l*) was used as the basal medium.

Production of indole was determined by using sea water broth supplemented with tryptophane (0.1%), and also SIM medium (Nissui Co.) prepared with 3% NaCl solution. Methylred (MR) and Voges-Proskauer (VP) tests were determined by using VP–MR medium (Nissui Co.), prepared with 3% NaCl solution. Indolepyruvic acid reaction (IPA) was determined by using BC test-IPA reaction medium (Nissui Co.). The production of catalase, oxidase and urease, nitrate reduction, fermentative and oxidative utilization of glucose were determined by the method described by COWAN[2] using sea water agar or broth as the basal medium.

Blackening of filter paper strips, soaked in lead acetate and suspended above sea water broth was taken to indicate H_2S production. The ability to utilize citrate was determined by using Christensen citrate agar medium and SC medium (Nissui Co.) prepared with 3% NaCl solution. The ability to produce acid from tartrate was determined by using Jordan tartrate agar medium (Nissui Co.) prepared with 3% NaCl solution. Arginine-alkaline reaction and the production of lysine and ornithine-decarboxylase were determined by using glucose sea water broth (Glucose 0.05%) supplemented with one of these amino acids and phenol red as an indicator. Incubation was carried out by using the test tubes covered with and without sterilized oil paraffin on top of the medium. These abilities and reactions were also confirmed by the method of Møller[2].

Degradation of gelatin, starch and chitin was determined by the method described by COWAN[2]. For testing the ability of the isolate to hydrolyze gelatin, starch or chitin, sea water agar medium was supplemented with 1% of gelatin, starch or chitin, respectively. By flooding the gelatin plate with several m*l* of a solution ($HgCl_2$ 15 g, HCl 20 m*l* and water 100 m*l*), unhydrolyzed gelatin will be coagulated to white opaque appearance. The colonies of hydrolyzing organisms will be surrounded by a clear zone. Starch plate was treated with Lugol's iodine solution[2]. The starch-hydrolyzing colonies will be surrounded by coloress zones. Colonies of chitin-decomposing bacteria will be surrounded by a clear zone. Gelatin-hydrolysis was also determined by liquefaction. A tube of solidified nutrient gelatin (Gelatin 12% and Sea water broth 1*l*) was inoculated the bacteria by stabbing a wire. After inoculation the liquefaction of gelatin was observed daily for 1 month.

Casein hydrolysis was determined by the method of Martley[3]. Tween-80 hydrolysis was determined by the method described by COWAN[2]. Action on milk was tested by using skimmed milk added by bromcresol purple

as an indicator.

Acid production from L-arabinose, D-xylose, D-glucose, D-fructose, D-galactose, D-mannose, sucrose, maltose, lactose, cellobiose, glycerol, D-mannitol, dulcitol, innositol, starch, inulin and salicin was assayed by adding the substances at a final concentration of 1% in semisolid sea water agar medium (Agar 0.3%). Acid and gas productions within 3 weeks were observed as positive results.

Resistance to antibacterial agents was determined by the disk method. The antibacterial substances included in the analyses were Vibrio static agent 0/129 (2, 4-diamino 6, 7-diisopropyl pteridine), Penicillin (20 U), Kanamycin (50 μg), Tetracycline (200 μg), Chloramphenicol (100 μg), Erythromycin (50 μg), Colistin (150 U), Novobiocin (20 U), Lincomycin (30 μg), Cephaloridine (30 μg) and Sulfisoxazol (400 μg).

Hemolysis was observed by using sea water agar plate supplemented with horse blood.

Results and Discussion

Morphological and Cultural Characteristics of the Isolate

Cells: Short rods, straight, 0.5 by 1.0–1.2 μ, occurring singly. Motile with a single polar flagellum. Gram-negative. Asporogenous. Agar colony: Good growth, circular, smooth, but later arborescent, convex, entire. Pigment is not produced. Agar slant: Good growth, filiform, butyrous, but later arborescent. Pigment is not produced. Liquid medium: Good growth, turbid, pellicle. Pigment is not produced. MacConkey agar: Good growth, attack of lactose. CVT agar: Good growth. Reduction of triphenyltetrazolium chloride. PEA agar: No growth. King A medium: Good growth. Pigment is not produced. King B medium: Good growth. Pigment is not produced. Anaerobic growth: Good growth under reduced oxygen condition (Facultatively anaerobic). Growth temperature: It grows at 5–30°C. No growth at 37°C. Growth in the presence of NaCl: It grows in 1–10%. No growth in 0%.

Biochemical Characteristics

Biochemical characteristics are shown in Table 1.

Table 1. Biochemical characteristics of the isolate

Characteristics	Remarks*
Indole	−
MR	−
VP	−
IPA	−
Catalase	+
Oxidase	+
Urease	−
Reduction of nitrate	−
Growth on OF medium	F
H_2S	−
Citrate utilization	+
Acid from tartrate	−
Arginine-alkaline	+
Lysine decarboxylase	+
Ornithine decarboxylase	+
Gelatin hydrolysis	+
Starch hydrolysis	+

Table 1. (continue)

Characteristics	Remarks*
Chitin hydrolysis	−
Casein hydrolysis	+
Tween 80 hydrolysis	+
Reaction to milk	Coagulation and peptonization
Acid (gas) production from	
Arabinose	−(−)
Xylose	−(−)
Glucose	+(−)
Fructose	+(−)
Galactose	+(−)
Mannose	+(−)
Sucrose	+(−)
Maltose	+(−)
Lactose	+(−)
Cellobiose	−(−)
Glycerol	−(−)
Mannitol	+(−)

Table 1. (continue)

Characteristics	Remarks*
Dulcitol	−(−)
Inositol	−(−)
Starch	−(−)
Inulin	−(−)
Salicin	−(−)
Hemolysis	−
Sensitivity to antibacterial substances	
0/129	+
Penicillin	−
Kanamycin	+
Tetracycline	+
Chloramphenicol	+
Erythromycin	+
Colistin	+
Novobiocin	−
Lincomycin	−
Cephaloridine	−
Sulfisoxazol	+

* +: Positive result −: Negative result

In order to identify the organism under investigation, the manuals for general bacteriological use were reffered[2,4-7]. The present unknown organism is Gram-negative rod, asporogenous, motile by means of a polar flagellum, facultatively anaerobic. It produces oxidase, catalase, and acid from glucose but no gas is formed. It grows on OF medium fermentatively. These characteristics correspond with those of a brief synopsis of the "*Vibrio* and *Vibrio*-like groups"[2]. Further it is sensitive to 0/129, grows in 6% NaCl, and does not hydrolyze chitin. It is obviously a member of the genus *Vibrio*. According to the systematic identification of psychrotroph described by VANDERZANI et al.[8], the present organism corresponds closely with the description of the genus *Vibrio*, since it is Gram-negative and sensitive to 0/129, grows on MacConkey agar and OF medium fermentatively, and does not produce yellow pigment.

In the Bergey's manual[4], the five species are described in the genus *Vibrio*, that is, *V. cholerae, V. parahaemolyticus, V. anguillarum, V. fisheri* and *V. costicola*.

In general features, *V. cholera* grows at 37°C and does not grow in 10% NaCl. It is negative for arginine-alkaline reaction. It produces indole. It is sensitive to Novobiocin.

The description of *V. cholerae* contains four biotypes, that is, *cholerae, eltor, proteus* and *albensis*, according to the Bergey's manual. The bacteriological characteristics of these four type cultures were compared with those of the present isolate. The different characteristics are as follows in addition to the general features. *Cholerae* type is positive to MR reaction. It reduces nitrate. It does not grow at 5°C and in 8% NaCl. It grows in 0% NaCl. *Eltor* type is positive to MR and VP reaction. It reduces nitrate. It does not grow at 5°C and in 8% NaCl. It grows in 0% NaCl. *Proteus* type does not decarboxylate lysine. *Albensis* type is positive to VP reaction and reduces nitrate. It does not grow at 5°C and in 7% NaCl. It does not produce acid from mannose. In conclusion, the present isolate can be differenciated from *V. cholerae* in the Bergey's manual.

V. parahaemolyticus produces indole. It grows at 37°C. It is negative to arginine-alkaline reaction. The description of *V. parahaemolyticus* contains two biotypes, that is, biotype I and II, according to the Bergey's manual. The bacteriological characteristics of these two type cultures were compared with those of the present organism. The different characteristics are as follows in addition to the general features. Biotype I is positive to MR reaction, does not grow in 10% NaCl, does not produce acid from sucrose. Biotype II is positive to VP reaction. According to these differences, the present isolate does not resemble this species.

V. anguillarum produces indole, does not utilize citrate. It grows in 0% NaCl and does not grow in 10% NaCl. It does not produce lysine and ornithine-decarboxylase. It is sensitive to Novobiocin. These differences make it clear that the present organism does not belong to *V. anguillarum* described in the Bergey's manual[4].

Some of the characteristics of the isolate are compared with those of four strains of *V. anguillarum* described by TAJIMA et al[9]. These strains are NCMB 6, NCMB 829, NOAA V-775 and NOAA V-1669 strains. Some bacteriological characteristics of the present organism differ from those of four strains of *V. anguillarum* as follows. These strains are sensitive to Novobiocin. They do not grow in 7% NaCl. They do not produce lysine and ornithine-decarboxylase. In addition to these differences, NCMB 6 strain is positive to VP reaction, produces indole.

It produces the hemolytic zone in blood-agar plate. NCMB 829 strain is positive to VP reaction and produces indole. It grows at 37°C. It produces the hemolytic zone in blood-agar plate. NOAA V-775 strain is positive to VP reaction. It grows at 37°C. It produces the hemolytic zone in blood-agar plate. NOAA V-1669 strain is positive to MR reaction, does not utilize citrate, and produces acid from salicin. It is negative to arginine-alkaline reaction. It does not hydrolyze Tween 80. Therefore, *V. anguillarum* described by TAJIMA et al[9]. must be also eliminated.

The present organism has several different characteristics from those of *V. fisheri* described in the Bergey's nanual. *V. fisheri* is positive to MR reaction and does not utilize citrate. It does not grow in 10% NaCl. It is negative to arginine-alkaline reaction. It does not produce ornithine-decarboxylase. It is sensitive to Novobiocin.

The description of *V. fisheri* contains *V. fisheri* NCMB 1281 strain proposed by HENDRIE[5] and also contains *V. marinus* MP-1 (ATCC 15381) and PS-207 (ATCC 15382) strains proposed by COLWELL[10]. The bacteriological characteristics of these type cultures were compared with those of the present organism. The different characteristics are as follows. These strains are positive to MR reaction. They produce acid from glycerol and starch, but do not produce acid from mannitol. They reduce nitrate. NCMB 1281 strain does not produce acid from lactose and sucrose. It produces luminescence. MP-1 strain does not produce acid from galactose, mannose, lactose and sucrose. It does not grow at 30°C, and does not grow in 5% NaCl. PS-207 strain does not liquefy gelatin. Finally, the present isolate can be differenciated from *V. fisheri* described in the Bergey's manual and also from *V. fisheri* NCMB 1281, *V. marinus* MP-1 and *V. marinus* PS-207 strains on the point of above mentioned.

V. costicola does not produce lysine and ornithine-decarboxylase. It does not hydrolyze starch and casein. It is sensitive to Novobiocin. Therefore, *V. costicola* will be also eliminated.

In conclusion, from the morphological, cultural and biochemical characteristics, the present isolate was classified into the genus *Vibrio*. However, it can be differenciated from all of five species described in the genus *Vibrio* according to the Bergey's manual[4]. Thus, polysaccharide-producing bacterium No. M-812 strain has been conventionally named *Vibrio* sp. M-812.

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