TAXONOMICAL STUDIES OF A POLYSACCHARIDE-
PRODUCING BACTERIUM FROM SEA CUCUMBER,
STICHOPOUS JAPONICUS (SELENKA)

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An extracellular polysaccharide-producing bacterium No. D-10 strain has been isolated from the intestinal content of sea cucumber, Stichopus japonicus (SELENKA). The isolate was a Gram-negative, asporogenous rod, straight or slightly curved, 0.8 x 1.0-1.3 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 differentiated 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 intestinal content of sea cucumber, Stichopus japonicus (SELENKA). Information concerning the microbial flora in the digestive tract of fish is abundant(1-4). The data support the notion that the microorganisms found in the intestinal content of fish are mainly the genus Vibrio and Aeromonas. However, the information presently available concerning the microbial flora in the digestive content of marine animals other than fish is scanty.

The purpose of this study is to identify taxonomically this polysaccharide-producing bacterium named No. D-10 strain.

Materials and Methods

Isolation Procedures

A bacterial strain was isolated from the intestinal content of sea cucumber, Stichopus japonicus (SELENKA),
which was collected at Seto Inland Sea. A loopful of the intestinal content 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\(^{(16)}\). Cell form was recorded by the simple stain technique of Loeffler\(^{(16)}\). Gram-reaction was examined by the modified method of Hucker\(^{(16)}\). 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\(^{(16)}\). A surer test was also performed by emulsifying some of the old culture of a agar slant growth in 5 ml 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 and CVT 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 an 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. Catalase, oxidase, urease, nitrate reduction, fermentative and oxidative utilization of glucose were determined by the method described by Cowan\(^{(17)}\) 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 H₂S 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 Moeller\(^{(17)}\).

Degradation of gelatin, starch and chitin was determined by the method described by Cowan\(^{(17)}\). For testing the ability of the isolate to hydrolyze gelatin, starch or chitin, sea water agar medium was supplemented with 1% of
gelatin, sarch or chitin, respectively. By flooding the gelatin plate with several ml of a solution (HgCl₂ 15 g, HCl 20 ml and water 100 ml), 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. The starch-hydrolyzing colonies will be surrounded by colorless 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%) 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. Tween-80 hydrolysis was determined by the method described by Cowan. 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, cellulose, glycerol, D-mannitol, dulcitol, inositol, 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 production within 3 weeks was 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-disopropyl 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 hore blood.

Results and Discussion

Morphological and Cultural Characteristics of the Isolate

Cells: Short rods, straight or slightly curved, 0.8 by 1.0-1.3 μ, occurring singly and in pairs. Motile with a single polar flagellum. Gram-negative. Asporogenous. Agar colony: Good growth, circular, smooth, convex, entire. Pigment is not produced. Agar slant: Good growth, filiform, butyrous. Pigment is not produced. Liquid medium: Good growth, turbid, pellicle. Pigment is not produced. MacConkey agar: Good growth, attack lactose. CVT agar: Good growth. Reduction of triphenyl-tetrazolium chloride. 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 1-8%. No growth in 0 and 9%. Optimum growth in 3-4%.

Biochemical Characteristics

Biochemical characteristics are shown in Table 1.

In order to identify the organism under investigation, the manuals for general bacteriological use were referred. 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". 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 Vanderzant et al., the present organism corresponds closely with the description of 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, the five species are described in the genus Vibrio, that is, V. cholerae, V.
Table 1. Biochemical characteristics of the isolate

<table>
<thead>
<tr>
<th>Characteristics</th>
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<tbody>
<tr>
<td>Indole</td>
<td>-</td>
<td>Mannose</td>
<td>-(-)</td>
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<tr>
<td>MR</td>
<td>-</td>
<td>Sucrose</td>
<td>+(-)</td>
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<td>VP</td>
<td>-</td>
<td>Maltose</td>
<td>+(-)</td>
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<tr>
<td>Catalase</td>
<td>+</td>
<td>Lactose</td>
<td>+(-)</td>
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<tr>
<td>Oxidase</td>
<td>+</td>
<td>Cellobose</td>
<td>-(-)</td>
</tr>
<tr>
<td>Urease</td>
<td>-</td>
<td>Glycerol</td>
<td>+(-) weak</td>
</tr>
<tr>
<td>Reduction of nitrate</td>
<td>-</td>
<td>Mannitol</td>
<td>+(-)</td>
</tr>
<tr>
<td>Growth on OF medium</td>
<td>F</td>
<td>Dulcitol</td>
<td>-(-)</td>
</tr>
<tr>
<td>H₂S</td>
<td>-</td>
<td>Inositol</td>
<td>-(-)</td>
</tr>
<tr>
<td>Citrate utilization</td>
<td>-</td>
<td>Starch</td>
<td>+(-)</td>
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<tr>
<td>Acid from tartrate</td>
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<td>Inulin</td>
<td>-(-)</td>
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<td>Arginine-alkaline</td>
<td>+</td>
<td>Salicin</td>
<td>-(-)</td>
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<tr>
<td>Lysine decarboxylase</td>
<td>-</td>
<td>Hemolysis</td>
<td>-</td>
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<td>Ornithine decarboxylase</td>
<td>+</td>
<td>Sensitivity to antibacterial</td>
<td>+</td>
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<td>Gelatin hydrolysis</td>
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<td>Substances</td>
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<td>Starch hydrolysis</td>
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<td>0/129</td>
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<tr>
<td>Chitin hydrolysis</td>
<td>-</td>
<td>Penicillin</td>
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<tr>
<td>Casein hydrolysis</td>
<td>+</td>
<td>Kanamycin</td>
<td>+</td>
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<tr>
<td>Tween 80 hydrolysis</td>
<td>+</td>
<td>Tetracycline</td>
<td>+</td>
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<tr>
<td>Reaction to milk</td>
<td>Peptonization</td>
<td>Chloramphenicol</td>
<td>+</td>
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<tr>
<td>Acid (gas) production from</td>
<td>-(-)</td>
<td>Erythromycin</td>
<td>+</td>
</tr>
<tr>
<td>Arabinose</td>
<td>-(-)</td>
<td>Colistin</td>
<td>+</td>
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<tr>
<td>Xylose</td>
<td>-(-)</td>
<td>Novobiocin</td>
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<td>Glucose</td>
<td>+(-)</td>
<td>Lincomycin</td>
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<td>+(-)</td>
<td>Cephaloridine</td>
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<tr>
<td>Galactose</td>
<td>+(-)</td>
<td>Sulfisoxazol</td>
<td>-</td>
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* + : Positive result  - : Negative result

parahaemolyticus, V. anguillarum, V. fisheri and V. costicola.

V. cholerae grows at 37°C and in 0% NaCl and also it is negative for arginine-alkaline reaction. It produces lysine-decarboxylase and produces indole. Therefore, V. cholerae will be eliminated. V. costicola grows in 10% NaCl, produces acid from mannose, does not produce ornithine-decarboxylase, and does not hydrolyze casein. Therefore, V. costicola will be also eliminated. V. parahaemolyticus produces indole, utilizes citrate, produces acid from mannose, and grows at 37°C. It is negative to arginine-alkaline reaction, but produces lysine-decarboxylase, hence the present organism does not resemble this species. V. anguillarum produces indole, produces acid from mannose, grows in 0% NaCl, and does not produce ornithine-decarboxylase. These differences make it clear that the present organism does not belong to V. anguillarum described in Bergey’s manual.[19]

Some of the characteristics of the isolate are compared with those of four strain of V. anguillarum described by TAJIMA et al.[22] 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. NCMB 6 strain is positive to VP reaction, produces indole, produces acid from mannose, utilizes citrate, does not grow in 7% NaCl, and does not produce ornithine-decarboxylase. It produces the hemolytic zone in blood-agar plate. NCMB 829 strain is positive to VP reaction, produces indole, produces acid from mannose, utilizes citrate, does not grow in 7% NaCl, grows at 37°C, and does not produce ornithine-decarboxylase. It produces the hemolytic zone in blood-agar plate. NOAA V–775 strain is positive to VP reaction, produces acid from mannose, utilizes
citrate, does not grow in 7% NaCl, grows at 37°C, and does not produce ornithine-decarboxylase. It produces the hemolytic zone in blood-agar plate. NOAA V-1669 strain is positive to MR reaction, produces acid from mannose and salicin, and does not grow in 7% NaCl. It is negative to arginine-alkaline reaction and does not produce ornithine-decarboxylase. It does not hydrolyze Tween 80. Therefore, V. anguillarum described by TAJIMA et al(22), must be lasso eliminated.

The present organism has several different characteristics from those of V. fischeri described in Bergey’s manual. V. fischeri is positive to MR reaction, produces acid from mannose. It is negative to arginine-alkaline reaction, produces lysine-decarboxylase and does not produce ornithine-decarboxylase.

The description of V. fischeri contains V. fischeri NCMB 1281 strain proposed by HENDRIE(20) and also contains V. marinus MP-1 (ATCC 15381) and PS-207 (ATCC 15382) strain proposed by COLWELL(23). The bacteriological characteristics of these three type cultures were compared with those of the present organism. The different characteristics are as follows. V. fischeri NCMB 1281 strain is positive to MR reaction, produces acid from mannose, but does not produce acid from lactose, sucrose and mannitol. It reduces nitrate and produces luminescence. V. marinus MP-1 strain is positive to MR reaction, but does not produce acid from galactose, lactose, sucrose and mannitol. It reduces nitrate. It does not grow at 30°C, and does not grow in 5% NaCl. V. marinus PS-207 strain is positive to MR reaction and does not liquefy gelatin. It produces acid from mannose and does not produce acid from mannitol. It reduces nitrate. Finally, the present isolate can be differentiated from V. fischeri described in the Bergey’s manual and also from V. fischeri NCMB 1281, V. marinus MP-1 and V. marinus PS-207 strains on the point of above mentioned.

In conclusion, from the morphological, cultural and biochemical characteristics, the present isolate was classified into the genus Vibrio. However, it can be differentiated from all of five species described in the genus Vibrio according to the Bergey’s manual and the other marine Vibrios. Thus, polysaccharide-producing bacterium No. D-10 strain has been conventionally named Vibrio sp. D-10.

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References


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