Physiological Properties of Clostridia Isolated from the Sediment in the Edo River Mouth

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One hundred and thirty eight strains of obligately anaerobic heterotrophs, genus *Clostridium*, were isolated from the sediment in the Edo River mouth using 5 different agar media, and examined for 19 physiological characteristics. Almost all strains produced proteolytic and saccharolytic enzymes and lecithinase. Few or none of the isolates produced cellulase, chitinase and lipase. Many organisms could grow in 0 to 2% NaCl and at 20°C to 37°C. Obligate halophiles were not isolated from the estuarine sediment. It is shown from the characteristics of casein hydrolysis, fermentation of lactose and sucrose and growth at 10°C that the clostridial composition varied with the temperature and/or with the medium to be isolated.

The isolates were classified into 13 groups based on 7 characteristics which are important in the taxonomy of genus *Clostridium*. Groups 3, 5 and 7 showed relatively high proportions of the isolates. Especially group 5 which seems to be *C. perfringens*, distributed widely in the sediment of the inner part of Tokyo Bay.

Although considerable information has been accumulated on the aerobic and facultatively anaerobic bacteria in ocean waters and sediments, there has been few investigations of obligate anaerobes with the exception of pathogenic bacteria.¹⁻⁴⁾ SUGITA *et al.*⁵⁾ investigated on the bacterial flora of the sediment in the Edo River mouth which is located in the inner part of Tokyo Bay from May 1980 to March 1981, and found that obligately anaerobic spore-forming heterotrophs, genus *Clostridium*, predominated with population density of 10³ to 10⁴ per gram of sediment. Clostridia are known to be present in marine and freshwater sediments.^{6,7)} However, the ecology of these bacteria in aquatic environments has yet not been clarified.

As the distribution of organisms is restricted by environmental factors, the investigation of physiological properties of isolates is of important to assume the ecology of these bacteria. In the present paper we shall describe the characteristics of clostridia isolated from the sediment in the Edo River mouth.

Materials and Methods

Isolation Procedure

Sediment samples were collected at stations 1, 2 and 3 in the Edo River mouth from December

1980 to September 1981 using SEKI's bottom sampler (Rigosha) as described previously.⁵⁾ Water samples were collected using KITAHARA's sampler (Rigosha). The sediment was diluted quantitatively with seawater diluent⁸⁾ and inoculated onto PYBG agar,⁹⁾ 1/20 PYBG agar,⁹⁾ N-PYBG agar,⁹⁾ GAM agar (Nissui) and FM-CW agar (Eiken). Inoculated PYBG, 1/20 PYBG, N-PYBG and GAM agar plates were incubated at 20°C for 5 days and FM-CW agar plate, at 37°C for 2 to 3 days, both under anaerobic condition. Anaerobiosis was established by evacuating the atmosphere of an anaerobic jar containing steel wool which was activated by an acidic cupric sulfate solution and replacing the atmosphere with CO₂ gas.⁹⁾

After incubation, colonies were divided into some types according to colonial morphology. Each representative colony type was plated on PYBG or 1/20 PYBG agar, and purified by a second streaking onto the same medium.

The purified bacteria were examined on the basis of Gram reaction, cellular morphology, aerobic growth and spore formation. The isolates identified as genus *Clostridium* were preserved in cooked meat medium (BBL) and/or chopped meat medium.¹⁰⁾ Both media were prepared with 1% NaCl solution instead of distilled water.

Physiological Tests

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Fig. 1. Physiological properties of clostridia isolated at 37°C (▨) and 20°C (□) from the sediment of the Edo River mouth.

For preparation of inocula, modified peptone-yeast extract-Fildes solution (modified PYF) broth¹¹⁾ with 0.1% glucose was used. The composition of modified PYF broth was as follows: Trypticase peptone (BBL), 5 g; Proteose peptone No. 3 (Difco), 5 g; Bacto-yeast extract (Difco), 10 g; Fildes peptic digest of blood (Fildes solution), 40 ml; mixture of 0.03% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.04% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1% K_2HPO_4 , 0.1% KH_2PO_4 and 1% of NaHCO_3 (salts solution), 40 ml; NaCl, 10 g; L-cysteine·HCl· H_2O , 0.5 g; distilled water, 920 ml; pH 7.6. The medium was autoclaved at 115°C for 20 min and, after cooling, was aseptically supplemented with 0.05 ml of ascorbic acid-cysteine solution per 3 ml immediately before use.

The abilities to hydrolyze casein, cellulose, starch, chitin, tributyrin and lecithin were examined on modified EG-F agar¹¹⁾ supplemented with 2% casein (NBC), 0.5% Avicel (Asahi Kasei), 0.5% starch, 5% precipitated chitin, 2% tributyrin or 10% egg yolk. The composition of modified EG-F agar was as follows: Lab-lemco powder (Oxoid), 2.4 g; Proteose peptone No. 3 (Difco), 10 g; Bacto-yeast extract (Difco), 5 g; Na_2HPO_4 , 4 g; NaCl, 10 g; Fildes solution, 40 ml; L-cysteine·HCl· H_2O , 0.5 g; Agar No. 1 (Oxoid), 10 g; distilled water, 960 ml; pH 7.6. The ability to hemolyze horse blood was examined on EG-F agar. The isolates were inoculated on test media, and incubated at 30°C in an anaerobic, steel wool jar with an atmosphere of 90% N_2 –10% CO_2 for 5 to 10 days. Hydrolysis of cellulose, chitin and tributyrin was recognized as a clear zone around colonies. To detect hydrolysis of casein, test plate was flooded with a 15% solution of mercuric chloride in HCl. Starch hydrolysis was determined by a

clearing of the medium around the colonies by flooding the plate with Lugol iodine solution.

Acid production from carbohydrates was determined in modified PYF broth containing 0.5% (w/v) of the carbohydrates to be tested. For the determination of sugar fermentation, pH value was measured directly in culture tube by a long combination electrode as recommended by HOLDEMAN *et al.*¹⁰⁾

Indole production was determined in indole-nitrate medium (BBL) supplemented with Fildes solution by adding KOVACS reagent.¹⁰⁾

The isolates were also tested for their abilities to grow in 0, 2 and 4% NaCl and at 10, 20, 30 and 37°C using EG-F agar as basal medium. L-Cysteine, NaCl and Fildes solution were omitted from EG-F agar to examine the growth in 0% NaCl.

Results and Discussion

The Edo River mouth with the water depths of 1.5 to 15.5 m, is a polluted area, and the anoxic water mass, called "Aoshio", was observed at the depth more than 10 m from June to September 1981. Some environmental factors in lower water from December 1980 to September 1981 had the following characteristics: Chlorinity, 5.00–14.89‰; water temperature, 9.2–27.7°C, dissolved oxygen, 0.0–9.2 ppm; chemical oxygen demand (COD), 0.2–2.5 ppm.

Total viable counts and viable counts of clostridia in the sediment were 10^8 g^{-1} and 10^8 to 10^4 g^{-1} , respectively, as same population densities as reported previously.⁹⁾ Of 138 strains, 76 and 62 strains were isolated at 37°C (FM–CW agar) and

Table 1. Grouping of clostridia isolated from the sediment in the Ebo River mouth, Tokyo Bay

Group	Gelatin	Lipase	Lecithinase	Indole	Glucose	Lactose	Sucrose	Isolates at	
								20°C	37°C
1	+	+	+	—	+	—	+	5*	0
2	+	—	+	+	+	+	+	0	2
3	+	—	+	+	+	—	—	12	1
4	+	—	+	+	—	—	—	1	0
5	+	—	+	—	+	+	+	10	55
6	+	—	+	—	+	—	+	4	2
7	+	—	+	—	+	—	—	17	12
8	+	—	+	—	+	+	—	0	1
9	+	—	—	—	—	—	—	2	1
10	—	—	+	—	+	+	+	6	1
11	—	—	+	—	+	+	—	1	0
12	—	—	+	—	+	—	—	1	0
13	—	—	—	—	+	+	+	3	1
Total								62	76

* Number of isolates.

20°C (other agars), respectively. Physiological properties of clostridia are shown in Fig. 1.

Almost all strains produced protease and liquefied gelatin and/or hydrolyzed casein. Few or none of isolates hydrolyzed cellulose, chitin and tributyrin. Thirty six to 76% of isolates hydrolyzed starch and 86 to 97% produced lecithinase. About a half showed β -hemolysis of horse blood. Almost all strains could utilize glucose, but fermentation of sucrose and lactose differed somewhat with the temperature at which the organism was isolated. Only a few strains produced indole. Many organisms could grow in 0 to 2% NaCl, and obligately halophilic bacteria were not isolated. Almost all strains could grow at 20 to 37°C, but 42 strains which could be isolated only at 20°C were able to grow at 10°C. It is suggested from these results that clostridial composition varied with the temperature and/or with the medium to be isolated. As water temperature of lower layer decreases to less than 10°C in winter, it must be important to clarify whether these organisms are present *in situ* with vegetative or dormant form.

Of 19 characteristics tested, gelatin liquefaction, production of lipase, lecithinase and indole, and fermentation of glucose, lactose and sucrose are known to be very important in the taxonomy of genus *Clostridium*. Therefore, 138 strains isolated from the sediment were divided into 13 groups based on 7 characters as shown in Table 1. Group 5 and 7 occupied in relatively high proportion among the strains isolated at 37°C, and in addition, group 3 also showed high percent among the

organisms isolated at 20°C. Although the proportion of group 5 was noticeable in the strains isolated at 37°C, this may be due to the medium used to isolate, which is a selective and differential medium for *Clostridium perfringens*.

According to the VPI manual,⁶⁾ group 5 seem to be *C. perfringens* or *C. sardiniensis*. These bacteria, especially the former is known to occur in polluted areas.^{2,10,12)} MATCHES and LISTON¹⁸⁾ isolated 244 strains of clostridia from sediments in Puget Sound and reported that most of these organisms were mainly composed of *C. perfringens* (37.3%), *C. bifermentans* (21.3%) and *C. novyi* (17.2%). SUGITA *et al.*¹⁴⁾ have isolated 42 strains of the clostridia from benthic animals and sediments in the Sanmaizu Bar, Tokyo Bay, and 76% of organisms seem to be group 5. Thus, group 5 are predominantly present in the polluted area such as the inner part of Tokyo Bay. As reported by WOOD and TRUST,¹⁵⁾ and MATCHES *et al.*,²⁾ the bacteria belonging to group 5 may have originated from sewage, freshwater sediments, soil and/or bird feces.

Although *C. oceanicum* and *C. stricklandii* are known to be originated from marine sediments^{16,17)} their salt requirement which should be an important characteristics in marine bacteria,¹⁸⁾ has yet not been clarified. DAILY *et al.*⁷⁾ studied obligate anaerobes in estuaries, and reported that the isolation of anaerobes was limited to the area with a relatively low salinity. Our results also show that halophilic clostridia were failed to be isolated. There are still problem whether a truly marine type of clostridia is present. Further study concerning

this problem should be performed.

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