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Isolation of Strictly Anaerobic Halophiles from the Aerobic Surface Sediments of Hypersaline Environments in California and Nevada

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1. SUMMARY

Four strictly anaerobic, chemoorganotrophic halophiles were isolated from the hypersaline surface sediments of the evaporating closed lagoon at the rim of Salton Sea, California, and of Big Soda Lake, Nevada, whose condition was not strictly anaerobic. All of the isolates were Gram-negative, motile, non-spore-forming, moderately halophilic eubacteria and required a minimum concentration of 3–10% NaCl in the growth medium. Among the four isolates, strain SS-21 could grow at more than 30% NaCl concentration, and strain M-20 was an alkalophile. Isolation of these bacteria suggests that a variety of anaerobic halophiles is widely distributed in hypersaline environments.

2. INTRODUCTION

Most studies of the bacteria which grow in extreme environments of high salt concentration have been focused on aerobic halophiles, and studies of anaerobic halophiles have been few. Now, only four species of obligately anaerobic, moderately halophilic, chemo-organotrophs are known and available for study [1]; *Haloanaerobium praevalens* [2], *Halobacteroides halobius* [3], *Sporohalobacter lortetii* [4], and *Sporohalobacter marismortui* [5]. These four strains were all isolated from the deep bottom sediments or the salt flat of hypersaline lakes (the Great Salt Lake, U.S.A. and the Dead Sea, Israel), whose condition was completely anaerobic. We have considered that halophilic anaerobes might live also in the surface sediments of the hypersaline environments, whose condition is not absolutely anaerobic. In this paper, we report the isolation of

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four strains of strictly anaerobic halophiles from the samples of the hypersaline surface sediments in California and Nevada, U.S.A.

3. MATERIALS AND METHODS

3.1. Source of organism

Hypersaline soil, mud, water and salts used in this study, were sampled at the hypersaline environments of California and Nevada, U.S.A., in March 1987. Salty samples were collected from surface sediments at about 0 to 10 cm depth of evaporation ponds and soda lakes and were kept and transported to Japan at room temperature in 50-ml screw-capped glass tubes with butyl rubber septums. These tubes contained the following basal mineral and nutrient solution (g/l); KH_2PO_4, 0.5; K_2HPO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.1; $(NH_4)_2SO_4$, 1; $CaCl_2 \cdot 2H_2O$, 0.1; resazurin, 0.001; Tween 80, 0.2; Bacto Agar (Difco), 0.5; L-cysteine · HCl, 0.5; $Na_2S \cdot 9H_2O$, 0.5; NaCl, 60.

3.2. Isolation and culture methods

All strains were isolated anaerobically as described by Balch et al. [6]. Each sample was incubated at 37 °C, in a 50-ml screw-capped glass tube with a butyl septum containing 10 ml of medium (g/l): NaCl, 234; KCl, 1; $MgSO_4 \cdot 7H_2O$, 2; $(NH_4)_2SO_4$, 0.5; glucose, 5; trypticase (BBL, Becton Dickinson and Co., Cockeysville, MD, U.S.A.), 5; yeast extract (Difco Laboratories, Detroit, MI, U.S.A.), 5; L-cysteine · HCl, 1; resazurin, 0.001; trace element solution and vitamin solution [6], 10 ml/l of each. The pH was adjusted to 7.5 with NaOH before autoclaving. When pH was adjusted to 10.0, Na_2CO_3 solution was added after autoclaving. The gas phase was N_2 or a mixture of N_2 and H_2 (8 : 1). Isolation of pure cultures was performed on agar media in plastic petri dishes in an anaerobic chamber (Forma Scientific, Marietta, OH, U.S.A.). For enrichment culture, pH and NaCl concentration were varied. Bacterial growth was followed by measuring optical density at 660 nm with a Bausch and Lomb Spectronic 21 spectrophotometer (Milton Roy Co., Rochester, NY, U.S.A.). The ability to utilize different carbon sources was tested by lowering the yeast extract and trypticase concentration to 0.05%, and replacing the glucose by the compound tested at a concentration of 5 gl/l. The NaCl concentration of the medium was 10% except for strain SS-21 of 15%.

3.3. Cell morphology

Cells were examined using a phase contrast microscope (Microphoto FX, Nikon Corp., Tokyo, Japan), after 12 h incubation of cultures at 37 °C using a medium of 10% NaCl, 0.5% glucose and pH 7.8, except for 20% NaCl concentration for strain SS-21 and pH 9.0 for strain M-20.

3.4. Analysis of G + C content of DNA

DNA was extracted and purified by the method of Saito and Miura [7]. DNA base composition was determined by reversed-phase high performance liquid chromatography after enzymatic hydrolysis of DNA into nucleosides by the method of Tamaoka and Komagata [8].

3.5. Analysis of fermentation products from glucose

Cells were grown in 50 ml anaerobic tubes containing 10 ml of medium with 0.5% glucose and 10% NaCl, except for strain 21 which was 15% NaCl. Fermentation products were assayed after 1 or 2 day incubation at 37 °C. Organic acids and alcohols were measured using a Shimadzu LC-6 high performance liquid chromatograph (Shimadzu Corp., Kyoto, Japan), and a column of Shodex Ionpak KC-811 (Showa Denko K. K., Japan) eluted with 0.1% H_3PO_4 at 60 °C. Lactate and pyruvate were also assayed enzymatically using test kits (Boehringer Mannheim GmbH, F.R.G.). Hydrogen and carbon dioxide were detected using a Shimadzu GC-15A gas chromatograph (Shimadzu Corp., Japan), equipped with a thermoconductivity detector, and a dual column of Molecular Sieve 5A (Union Carbide, U.S.A.) and Shimalite Q (Shimadzu Corp., Japan) plus Porapak Q (Waters, Milford, MA, U.S.A.) operated at 60 °C with nitrogen as carrier gas.

4. RESULTS

4.1. Isolation of anaerobic halophiles from Salton Sea, California and Big Soda Lake, Nevada

Three anaerobic halophiles (strains SS-11, SS-15 and SS-21) were isolated from 0–10 cm depth of surface sediment of hypersaline muds in the closed lagoon at the south east rim of Salton Sea (near the Rock Hill in Salton Sea National Wildlife Refuge; at the sampling site, pH and salt concentration of water were 8.3 and 15%), in a screening medium of 23.4% of NaCl concentration. Strain M-20 was isolated from the surface sediments in Big Soda Lake, Nevada (north west from Fallon; at the site, pH and salt concentration of water were 9–10 and 10–15%), using a screening medium of pH 10 and with 20% NaCl concentration. Enrichment liquid cultures were transferred to anaerobic solid medium on an agar plate, and single colonies were selected. Stock cultures were kept in a 10% glycerol solution at −80 °C. Under these conditions viability was maintained for more than one year.

4.2. Cellular properties

All four strains were Gram-negative, non-spore-forming, strictly anaerobic rods and exhibited motility by means of peritrichous flagella. Cells of strain SS-11 were short straight rods, and appeared as single or paired rods with dimensions 1.0–1.3 × 2.5–3.5 μm. Strain SS-15 was a long straight rod which also appeared singly or in pairs, with dimensions 0.6–0.8 × 3.0–8.0 μm. Strain SS-21 was a straight or a slightly curved rod and appeared as single, paired rods, or as long chains, with cell dimensions 0.5–0.7 × 2.5–4.0 μm. Strain M-20 cells were small rods with dimensions 0.5–0.7 × 0.9–2.2 μm. In cell membranes of all four isolates, fatty acid glycerol esters were found, so these isolates were not archaebacteria but belonged to the eubacteria. DNA base compositions of strains SS-11, SS-15, SS-21 and M-20 were 30.3, 30.5, 34.9 and 31.1 mole % guanine plus cytosine.

4.3. Growth properties

The optimum temperature for growth of the strains was between 37–45 °C, and no growth was observed above 50–55 °C. The optimum range of

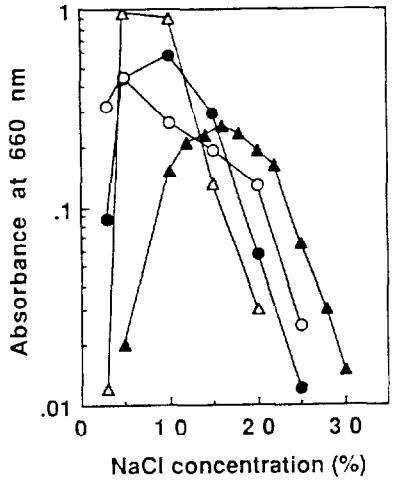

Fig. 1. Effect of NaCl concentration on the growth of isolated halophilic anaerobes. ●: strain SS-11. ○: strain SS-15. ▲: strain SS-21. △: strain M-20.

NaCl concentration for growth of strains SS-11, SS-15 and M-20 was between 5 and 10%, and for strain SS-21 was between 12 and 20% (Fig. 1). The pH range for growth of the three isolates from Salton Sea was between 6.5 and 8.5–9.5, and the pH optimum was around 7.5 to 8.0 (Fig. 2). The growth pH of strain M-20 ranged from 7.0 to 10.0, and the optimum pH was between 8.5–9.5 (Fig. 2). Many different organic compounds stimulated

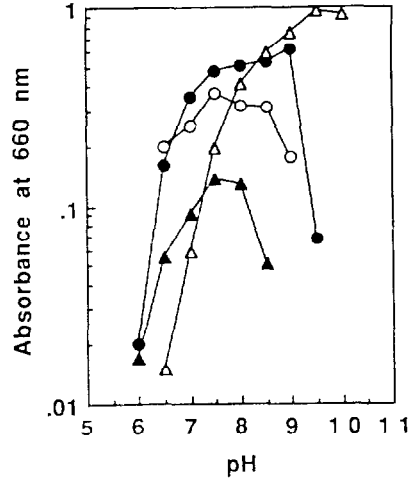

Fig. 2. Effect of pH on the growth of isolated halophilic anaerobes. ●: strain SS-11. ○: strain SS-15. ▲: strain SS-21. △: strain M-20.

Table 1

Stimulation effect of organic compounds on the growth of halophilic anaerobes isolated from the hypersaline environments in California and Nevada

Substrate	Strain isolated			
	SS-11	SS-15	SS-21	M-20
L-arabinose	−	−	+	−
D-ribose	−	−	+	+
D-xylose	−	−	+	−
D-fructose	+	+	±	+
D-galactose	−	±	+	+
D-mannose	−	+	+	+
sucrose	−	−	+	+
maltose	−	+	+	+
cellobiose	−	−	+	+
lactose	−	−	+	+
trehalose	−	−	+	+
melibiose	−	−	+	+
raffinose	−	−	±	−
D-gluconate	−	±	−	+
N-acetylglucosamine	+	−	+	+
glycerol	−	−	−	+
D-mannitol	−	−	+	−
glycine	+	−	−	−
L-glutamate	+	+	−	−
L-lysine	−	+	−	+

growth of the four isolates. Glucose, dextrin and soluble starch stimulated growth of all four strains significantly, whereas, L-sorbose, L-rhamnose, dextran, D-sorbitol, L-alanine, L-arginine, acetate, lactate, fumarate and succinate were not fermented by all four isolates. The other compounds tested showed different patterns as indicated in Table 1.

4.4. Fermentation products from glucose

Main fermentation products from glucose produced by strains SS-11, SS-15 and M-20 were acetate, propionate, *n*-butyrate, hydrogen and carbon dioxide, and strain M-20 also produced a small amount of lactate, whereas, fermentation products by strain SS-21 were ethanol, acetate, lactate, pyruvate, hydrogen and carbon dioxide.

5. DISCUSSION

Strictly anaerobic halophiles, so far reported, have been isolated from strictly oxygen-limiting environments; the anaerobic black sediments of the deep bottom of the Dead Sea [3,4], the Great Salt Lake [2] or on the shore of a hypersaline lake [5]. We isolated four anaerobic halophiles (strains SS-11, SS-15, SS-21 and M-20) from the hypersaline surface sediments of an evaporating closed lagoon and a soda lake, in California and Nevada, U.S.A., whose condition was not absolutely anaerobic. In the extreme environments of high salt concentration, oxygen solubility is limited [9]. At surface sediments in contact with air, oxygen concentration is considered to be low but to be enough to support the growth of aerobic species. On the other hand, the microenvironment inside of sediment particles is believed to be anaerobic as occurs in usual soil environments.

All four isolates were Gram-negative, non-spore-forming, strictly anaerobic, moderately halophilic eubacteria, similar to previously described species, *Haloanaerobium praevalens* [2] and *Halobacteroides halobius* [3]. Among the halophilic heterotrophic anaerobes, only *Haloanaerobium praevalens* was known to grow at more than 20% NaCl concentration, whereas all of our isolates could grow at this condition, and especially strain SS-21 could grow at 30% NaCl. Morphologically, strains SS-11 and M-20 were similar to *Haloanaerobium praevalens* except for motility, but strain SS-21 cells looked different from our three other isolates and other previously described species. Fermentation product from glucose of strains SS-11, SS-15 and M-20 were similar to that of *Haloanaerobium praevalens* with major products of acetate, propionate and *n*-butyrate, except that strain M-20 produced lactate also. The fermentation products of strain SS-21 were ethanol, acetate, lactate and pyruvate, which was different from both *Haloanaerobium praevalens* and *Halobacteroides halobius* [1–3]. Also the G + C content of DNA of strain SS-21 was higher than that of the other anaerobic halophiles. These observations indicate that a diversity of types of strict anaerobes live in such extreme environments.

Using the same hypersaline samples, when the salt concentration in the first screening medium was decreased to 5%, more variety of anaerobes was observed (data not shown). This observation shows that a lot of halophilic or halotolerant

anaerobes were living inside of the hypersaline particles. The discovery of these new halophilic anaerobes in the surface sediments of inland evaporating environments and a soda lake, confirms that strictly anaerobic halophiles are distributed universally in the hypersaline environments, the same as aerobic halophiles. In an ecological study, Oremland et al. [10] described a moderately halophilic, alkalophilic methanogen from Big Soda Lake, Nevada, where strain M-20 was isolated, and also haloalkalophilic methanogens were isolated from the same Salton Sea samples that we used (N. Nakatsugawa, personal communication). Zeikus et al. [2] have reported that in the Great Salt Lake, *Haloanaerobium praevalens* has a role as a producer of substrates for methanogenesis. So, our four anaerobic heterotrophic isolates may be participating in the recycling of nutrients in their environment together with halophilic methanogens.

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