Characterization of Anaerobic Cellulolytic Bacteria Isolated from Marine Environments

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Eleven strains of anaerobic bacteria capable of digesting cellulose have been isolated from marine environments. These isolates were Gram negative, non-spore-forming, rod-shaped bacteria, and could be divided into three groups: six strains of a yellow-pigmented group; three strains of a non-pigmented group; and two strains of a light brown-pigmented group. For characterizing the isolates detailed examinations were carried out with representative isolates: yellow-pigmented strain Y; light brown-pigmented strain B; and non-pigmented strain W. No essential difference was found between these strains in respect to biochemical reactions, inorganic requirements, or major organic acid accumulated in the cultures. Some differences were noted in the spectra of carbon and nitrogen sources as well as in the patterns of vitamin requirements. The strains did not correspond with any previously described species, and could probably be considered as new species belonging to genus *Bacteroides*.

There were a relatively large number of anaerobic cellulolytic bacteria in recent marine sediments especially in coastal regions of the sea¹⁾, and the biological degradation of cellulose by the organisms seemed unable to be disregarded in view of the carbon cycle of the coastal sea.

A survey of some literatures revealed that no detailed information is available on the characteristics of the anaerobic cellulolytic bacteria. Therefore, attempts were made to isolate anaerobic cellulolytic bacteria of marine origin, and the representative strains purely isolated have been characterized by morphological, biochemical, and cultural features. The present paper describes the results of this investigation.

Methods

Media

The medium used for isolation and maintenance (referred to as medium 1) was identical in composition to that reported earlier¹⁾. The same medium without 0.2% cellobiose and filter paper (referred to as medium 2) was employed to clarify the influences of sulfide concentration and temperature on growth. The basal medium used for most of the tests for nutritional requirements (referred to as medium 3) contained the following ingredients (g/l): NaCl, 24 g; MgCl₂·6H₂O, 11 g; Na₂SO₄, 4 g; CaCl₂, 1.5 g; KCl, 0.5 g; NH₄Cl, 2 g; FeCl₃, 5 mg; Na₂HPO₄, 10 mg; Na₂CO₃, 4 g; Na₂S·9H₂O, 0.5 g; vitamin-free casamino acids

(Difco), 0.5 g; cellobiose, 2 g; cysteine·HCl·H₂O, 0.5 g; *p*-aminobenzoic acid, 1 mg; biotin, 10 µg; cobalamin, 10 µg; choline·HCl, 1 mg; folic acid, 10 µg; niacin, 1 mg; Ca-pantothenate, 1 mg; pyridoxal, 1 mg; riboflavin, 1 mg; thiamine, 1 mg. The first 5 components of the medium mentioned above were added to make artificial seawater. For the inorganic requirement tests of strains B and Y, casamino acids-free medium 3 (referred to as medium 4) was employed. Medium 1 enriched with casamino acids (0.5 g/l) and biotin (10 µg/l) in place of peptone (referred to as medium 5) was employed to obtain a growth curve and some additional information during growth.

These media were dispensed under a gas phase of oxygen-free CO₂ in 10 ml amounts in 16 mm wide acid-cleaned borosilicate test tubes, capped with butyl rubber plugs and sterilized at 120°C for 20 min.

Isolation

An appropriate dilution of marine mud was inoculated into medium 1, and incubated at 30°C. The tube showing visual digestion of filter paper was selected, and transferred to molten medium 2 enriched with 1.5% agar and 0.5% acid-treated cellulose.²⁾ Then the inoculated tube was rolled in cold water until the medium was set in a thin layer on the tube wall, and incubated at 30°C. The colony which showed a clear area was transferred to medium 1 to ensure filter paper disintegration. The isolate was further purified by

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repeating the procedures described above.

Routine Tests for the Identification of Bacteria

The isolates were characterized biochemically by routine tests according to the Anaerobe Laboratory Manual³¹, but the media employed here were prepared by using aged seawater, and enriched with equivalent amount of cellobiose in place of glucose.

Growth Experiment

About 0.1 ml of actively-growing culture was employed as inoculum. Inoculation was done under a gas phase of oxygen-free CO₂, and incubation was carried out at 30°C for 20 days. The growth yields were determined by measuring the optical density at 660 m μ in a cuvette of 10 mm light-path. In some cases a few droplets of 6 N HCl were added to a culture for dissolving inorganic precipitates at the end of cultivation.

Chemical Analyses

Protein was determined according to the method of LOWRY *et al.*⁴¹ Sugar was determined by the anthrone method⁵¹. Organic acids were extracted with ether and analyzed in the same manner as described by CARLSSON⁶¹.

Bacterial Counting

Bacterial counting was carried out in the same manner as described by LUMPKINS and ARVESON⁷¹.

Measurement of Eh

Redox potential (*Eh*) of medium was measured by using redox meter just before inoculation.

Results and Discussion

Eleven strains of non-spore-forming, Gram negative rods, capable of digesting cellulose were purely isolated from marine sediments. Based on preliminary experiments, these isolates were divided into the following 3 groups: 6 strains of a yellow-pigmented group; 3 strains of a non-pigmented group; and 2 strains of a light brown-pigmented group. In most anaerobic marine environments the yellow-pigmented group was predominant and the light brown-pigmented or the non-pigmented group was found sporadically. The yellow-pigmented strains grew well and showed active digestion of cellulose. The light brown-pigmented strains grew slowly and the digestion of cellulose was not so rapid as compared with the yellow-pigmented strains. The non-pigmented strains showed very slow and scanty growth, and the strains did not digest cellulose completely unless a microbial contamination occurred in the medium.

The morphological, cultural, and biochemical characteristics of the representative strains belonging to each group are described in Table 1.

There are many reports dealing with the pure strain of anaerobic cellulolytic bacteria isolated

Table 1. Morphological, cultural, and biochemical properties of representative strains of marine cellulolytic anaerobes

Property	Light brown-pigmented strain B	Yellow-pigmented strain Y	Non-pigmented strain W
Morphological characteristics			
Form	Rod with round ends	Rod with round ends	Rod with round ends
Size	0.7 μ $\times$ 3–7 μ	0.7 μ $\times$ 3–7 μ	0.5 μ $\times$ 2–7.5 μ
Motility	Motile	Non-motile	Non-motile
Gram stain	Negative	Negative	Negative
Cultural characteristics			
Seawater cellobiose agar slant	Smooth, glistening, beaded	Smooth, glistening, filiform	Smooth, glistening, effuse
Filter paper degradation	Fiber separated on slight agitation	Fiber separated on slight agitation	Macerative rather than lytic
Biochemical characteristics			
Gelatin digestion	Slow liquefaction	Slow liquefaction	—
Nitrate reduction	Negative	Negative	Negative
Voges-Proskauer test	Negative	Negative	Negative
Indol production	Negative	Negative	Negative
Catalase	Negative	Negative	Negative

from intestine⁸⁾, rumen^{9,10)}, soil^{10,11)}, and sewage sludge¹⁰⁾, and simplified descriptive charts of some marine cellulolytic anaerobes were included in

a paper by LISTON¹²⁾. The new isolates, strains B, W, and Y, seem to be somewhat different from the cellulolytic anaerobes described in the early reports. The taxonomic position of the isolates will be discussed later.

The effects of varying concentrations of $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ on the growth of strains B, W, and Y are shown in Fig. 1. The approximate levels of *Eh* of the media are also illustrated in the same figure. Detectable growth of these strains was found only when the concentration of $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ was higher than 0.03%; the upper limits of *Eh* to permit the growth of these strains were in the range of -100 to 0 mV. That is, these strains could grow only in the media showing complete decolouration of resarfin. Therefore, these strains are fastidious in their requirements for anaerobiosis just like most strains of cellulolytic rumen bacteria.

The effects of temperature on the growth of strains B, W, and Y are shown in Fig. 2. These strains were all mesophilic organisms showing the maximal growth rate at *ca.* 30°C, and appreciable growth of strains B and Y was detected in the range of 20° to 30°C, but detectable growth of strain W occurred within the range of 20° to 45°C.

The growth yields of strains B, Y, and W at varying strengths of artificial seawater in medium 3 are shown in Fig. 3. The extreme concentrations of artificial seawater which allowed detectable growth of these strains were 25% and 200%. The maximal growth occurred at normal concentration of artificial seawater. Therefore, these strains seem to be placed in the same category with slight halophile which occurred ubiquitously in marine environments.

The growth yields in modified medium 4 (for strains B and Y) or medium 3 (for strain W) each of which contained reduced concentrations of an inorganic ingredient are shown in Table 2. Strictly speaking, the inorganic requirement of strain W was not conclusive due to the presence of inorganic components in casamino acids. But significant reduction of growth yields of each test strain was observed by lowering the added concentration of NaCl, MgCl_2 , CaCl_2 , KCl, or Na_2HPO_4 , but FeCl_3 and Na_2SO_4 could be omitted from the test media without interfering with the cell yields. Anyhow, so far as these data were concerned, sodium, magnesium, calcium, potassium, and phosphate ions seem to be essential for the growth of these three strains. The requirement of such ions is not so unique for marine bacteria.

Fig. 1. Effects of the concentrations of reducing agent on the growth of anaerobic cellulolytic bacteria. Symbols: ○, strain B; □, strain W; ●, strain Y; ▲, oxidation-reduction potential.

Fig. 2. Effects of incubation temperatures on the growth of anaerobic cellulolytic bacteria. Symbols: ○, strain B; ●, strain Y; □, strain W.

Fig. 3. Effects of the strengths of artificial seawater on the growth of anaerobic cellulolytic bacteria. Symbols: ○, strain B; □, strain W; ●, strain Y.

Table 2. Growth yields of marine anaerobic cellulolytic bacteria in the media having reduced concentrations of inorganic components

Component	Concentration added (g/l)	Growth, O.D. at 660 m μ		
		Strain B	Strain W	Strain Y
NaCl	2.4	0.084	0.017	0.149
	0.24	0.087	0.015	0.092
	0	0.088	0.015	0.083
MgCl ₂ ·6H ₂ O	1.1	0.208	0.023	0.187
	0.11	0.027	0.012	0.061
	0	0.002	0.016	0.004
Na ₂ SO ₄	0.4	0.193	0.027	0.198
	0.04	0.172	0.025	0.200
	0	0.186	0.026	0.185
NH ₄ Cl	0.2	0.101	—	0.188
	0.02	0.065	—	0.084
	0	0.018	—	0.022
CaCl ₂	0.15	0.310	0.022	0.187
	0.015	0.260	0.015	0.174
	0	0.009	0.016	0.007
KCl	0.05	0.125	0.021	0.161
	0.005	0.027	0.017	0.013
	0	0.022	0.017	0.013
Na ₂ HPO ₄	0.001	0.055	0.019	0.039
	0.0001	0.021	0.018	0.015
	0	0.010	0.016	0.019
FeCl ₃	0.0005	0.200	0.026	0.184
	0.00005	0.204	0.028	0.185
	0	0.188	0.028	0.201
Control (complete)		0.203	0.028	0.192

The availability of organic compounds as the sole source of carbon and energy was examined by using medium 3 as basal medium. The results are described in Table 3. The growth of strain B was supported at the expense of xylose, glucose, cellobiose, or maltose. The growth of strain W occurred on formate, acetate, or cellobiose, but strain Y could utilize only cellobiose out of twenty organic compounds tested. The addition of peptone, yeast extract, or beef extract in the concentration of 0.1% had no essential influence on the spectra of carbon and energy sources.

Considering the ubiquitous occurrence of cellulolytic anaerobes in coastal sediments, the restricted spectra are of interest for indirect evidence that coastal sediments are contaminated so universally and successively by cellulosic materials.

The availability of nitrogen compounds as a sole source of nitrogen was examined with medium 3 as basal medium. The results are shown in Table 4. Heavy or appreciable growth of strains B and Y was obtained with NH₄Cl, asparagine, peptone, tryptone, or casamino acids, and little or no growth

Table 3. Carbon sources utilized for the growth of marine anaerobic cellulolytic bacteria

Carbon source (5 g/l)	Growth, O.D. at 660 m μ		
	Strain B	Strain W	Strain Y
Acetate	0.006	0.009	0.000
Citrate	0.012	0.004	0.002
Formate	0.010	0.011	0.001
Fumarate	0.013	0.002	0.004
Lactate	0.013	0.004	0.002
Propionate	0.013	0.001	0.000
Pyruvate	0.006	0.002	0.001
Cellobiose	0.071	0.025	0.456
Fructose	0.013	0.001	0.004
Galactose	0.022	0.004	0.002
Glucose	0.046	0.004	0.002
Lactose	0.018	0.004	0.002
Maltose	0.030	0.000	0.002
Mannose	0.014	0.004	0.000
Saccharose	0.011	0.000	0.000
Starch	0.013	0.001	0.004
Xylose	0.051	0.001	0.003
Glycerol	0.010	0.000	0.000
Mannitol	0.009	0.004	0.003
Sorbitol	0.013	0.003	0.004
None	0.014	0.004	0.003

Table 4. Nitrogen sources utilized for the growth of marine anaerobic cellulolytic bacteria

Nitrogen source (2 g/l)	Growth, O.D. at 660 m μ		
	Strain B	Strain W	Strain Y
NaNO ₂	0.014	0.016	0.011
NaNO ₃	0.015	0.012	0.012
NH ₄ Cl	0.146	0.018	0.159
Asparagine	0.079	0.018	0.051
Na-glutamate	0.013	0.021	0.013
Casamino acids (Difco)	0.181	0.035	0.081
Peptone (Difco)	0.058	0.080	0.036
Tryptone (Difco)	0.064	0.065	0.030
None	0.016	0.018	0.013

took place with NaNO₂, NaNO₃, or Na-glutamate. On the other hand, lower but still appreciable growth of strain W was supported only with complex nitrogen sources such as tryptone, casamino acids, and peptone.

Attempts were made to determine the vitamin requirements of strains B, W, and Y by omitting individual vitamins from medium 3. The results are shown in Table 5. Strains B and Y uniformly required biotin for good growth, and there was no evidence of requirements for other vitamins, and 1 μ g/l of biotin was enough to give reproducible heavy growth for these strains. Whereas,

Table 5. Vitamin requirements of marine anaerobic cellulolytic bacteria

Vitamin	Requirement		
	Strain B	Strain W	Strain Y
<i>p</i> -Aminobenzoic acid	—	+	—
Biotin	+	+	+
Cobalamin	—	—	—
Choline	—	—	—
Folic acid	—	+	—
Niacin	—	—	—
Pantothenic acid	—	—	—
Pyridoxal	—	+	—
Riboflavin	—	—	—
Thiamine	—	—	—

Symbols: +, essential or stimulatory for growth; —, not essential.

the growth of strain W was stimulated by *p*-aminobenzoic acid, biotin, folic acid, and pyridoxal.

Typical growth curves for strains B and Y on medium 5 are illustrated in Fig. 4. The weight losses of cellulose and the accumulations of soluble protein and sugar during growth are also shown in the same figure. The continuous but slow growth of strains B and Y was observed in the first 15–20 days of incubation. Thereafter no appreciable

growth occurred by further incubations. On the other hand, discernible losses of cellulose and significant accumulations of soluble protein and sugar were noted after the first 12–14 days of incubation. Thereafter the losses of cellulose and the accumulations of soluble protein and sugar showed continuous increases. Soluble protein in the medium should include excreted cellulase, and the levels of soluble protein seemed to be roughly correlated with the losses of cellulose.

On the other hand, the accumulated sugar was regarded as a transient metabolite and seemed to be converted gradually into final products such as organic acids and CO₂. In fact, as shown in Fig. 5, organic acids were detected in the cultures. Irrespective of the test strain, the major organic acid detected by the chromatographic method was acetic acid. The fact may serve as an important adjunct in characterizing the isolates.

Taking all the available data into account, the isolated strains did not correspond with any previously described species^{3,13)}, and the strains could probably be considered as new organisms belonging to genus *Bacteroides*^{3,14)}, but the detailed characteristics of the strains are not identical to those cited in the descriptions of genus *Bac*-

Fig. 4. Growth curves of marine anaerobic cellulolytic bacteria and accumulation of sugar, excretion of protein, and loss of cellulose in the course of the growth. Symbols: ▲, bacterial count; △, loss of cellulose; ■, sugar; □, protein.

Fig. 5. Chromatograms of organic acids accumulated in the cultures of marine anaerobic cellulolytic bacteria. Symbols: A, acetic acid; iB, isobutyric acid.

teroides^{3,13}. Especially the fact that no accumulation of succinic acid occurred in the cultures of strains B, W, and Y seems to be important. Therefore, further studies are needed to draw a final conclusion as to whether strains B, W, and Y belong to genus *Bacteroides* or not.

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