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Characterization of an Alginolytic Marine Bacterium from Decaying Rishiri-kombu *Laminaria japonica* var. *ochotensis*

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An alginolytic marine bacterium, H-4, was isolated from decaying thalli of *Laminaria japonica* var. *ochotensis*. Strain H-4 is a gram-negative polarly flagellated rod, which oxidatively produces acid from glucose, is oxidase positive and requires seawater for growth. DNA base composition measured from melting point of DNA is 39.8 mol% G+C. According to these characteristics, the strain was identified as genus *Alteromonas*. *Alteromonas* sp. H-4 resembles *A. espejiana* but differs in DNA base composition and some properties and could not be identified to any species in the genus *Alteromonas*.

The productivity of extracellular alginate lyase by *Alteromonas* sp. H-4 increased with seawater concentration and decreased with casitone concentration in the media. Its productivity was observed in a medium without alginate. The bacterium produced a maximum amonut of alginate lyase in liquid media containing 75% seawater, 0.5% casitone, and 0.1% sodium alginate after 96 h incubation with shaking at 25°C.

In 1985, a disease which decayed in holes on the center of thalli of cultured Rishiri-konbu *Laminaria japonica* var. *ochotensis*, so called "*Anaaki-sho*", was observed, and the kombu crop was badly damaged. The disease has been suggested to be caused by bacterial pathogens, because the location and size of holes were different from the holes due to feeding by marine molluscs (personal communication). Also, the involvement of alginolytic bacteria in this disease was highly suspect since the cell walls of Laminariales are mainly composed of alginate.

A bacterium, designated strain H-4. with high ability to hydrolyze sodium alginate was isolated from decayed thalli of Rishiri-kombu. In this study, we characterized the strain H-4 and investigated the production conditions of alginate lyase.

Materials and Methods

Isolation and Identification of the Bacterium

Decayed thalli of Rishiri-kumbu were collected from coastal regions of Rishiri Island in Hokkaido. The samples were washed three times in sterilized natural seawater and homogenized by a stomacker (Colworth Co.) in sterile seawater. Homogenized samples were plated on a medium containing casitone (Difco) 2.0 g, yeast extract (Difco) 1.0 g, sodium alginate 1.0 g, agar 13.0 g, natural seawater 1,000 m*l*, pH 7.5 (CYS medium). Plates were incubated at 20°C for 7 days. Seventeen colonies showing different morphology were picked out and inoculated in a medium containing an autoclaved strip of makombu *Laminaria japonica*. After incubation at 20°C for 7 days, culture medium showing complete degradation of the strip were plated on CYS medium, and colonies were picked up and purified by streaking on the same medium. Of the four strains obtained, strain H-4 which showed most active degradation for makonbu thallus was used in the study.

Cell morphology, physiological and biochemical characteristics of strain H-4 were determined by the conventional methods.[1-3]

For observation of flagellum, fresh cultured cells were transferred to a colloid ion-coated grid, stained with 2% uranyl acetate, and were examined with a Hitachi transmission electron microscope (H-300).

The DNA of the bacterium was extracted by Marmur's method,[4] the melting temperature of the DNA was measured with a Hitachi spectrophotometer (type 202-20) and the base composition of DNA was calculated by Marmur &

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Doty's formula.[5]

The bacterium was identified according to Baumann *et al.*[3]

Effect of Medium Components for Production of Alginate Lyase

Growth and alginate lyase production of strain H-4 were observed in media with varying concentrations of seawater, casitone, and sodium alginate and were incubated with shaking at 25°C. From each culture medium, 3 m*l* was periodically taken out. Growth of the bacterium was determined by measuring turbidity at 620 nm using a Hitachi spectrophotometer (type 124), and after centrifugation (20,000×g, 30 min), alginate lyase activity in supernatant was measured by the method presented as follows.

Assay for Algingte Lyase Activity

Alginate lyase activity was measured by an increase in the absorbance at 235 nm with a Hitachi spectrophotometer by modified Tsujino and Saito's method.[6] The following conditions were used for the enzyme reactions: substrate (0.1% sodium alginate) in 0.1 M Tris-HCl (pH 7.5), 2.7 m*l*; and enzyme solution, 0.3 m*l*. The reaction mixture was incubated for 30 min at 30°C. One unit of enzyme activity was defined as increase of 0.01 optical density at 235 nm per min.

Results

Identification of Strain H-4

The strain H-4 was isolated from hole parts on thalli of diseased *Laminaria japonica* var. *ochotensis*. The bacterium strongly degraded sodium alginate, CM-cellulose, and thallus of *L. japonica*.

Characteristics of the strain H-4 are shown in Table 1. Strain H-4 was gram negative, polarly flagellated rod (Fig. 1) with a G+C content

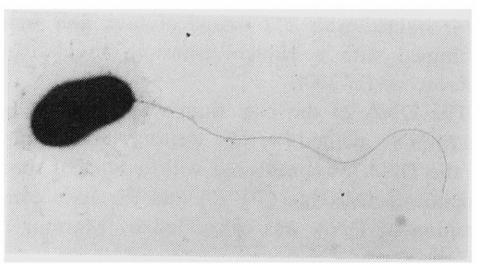

Fig. 1. Electronmicograph of *Alteromonas* sp. H-4. (×10,000).

Table 1. Characteristics of isolated strain H-4 and *Alteromonas espejiana*[3]

Characteristics	H-4	*A. espejiana*
Gram stain	−	−
Cell shape	st.	st.
Fragellar arrangement	pol.	pol.
PHB accumulation	−	−
Seawater requirement	+	+
OF test	O	O
Oxidase	+	+
Luminescence	−	−
Growth at:		
4°C	+	−
35°C	+	d
40°C	−	−
Reduction of NO_3^- to NO_2^-	−	−
Requirement for organic growth factor	−	+
Production of:		
Amylase	+	+
Gelatinase, lipase	+	+
Alginase	+	+
Chitinase	−	−
Utilization of:		
D-Mannose	−	d
D-Galactose	+	+
D-Fructose	+	d
Sucrose	+	+
Maltose	+	+
Cellobiose	+	d
Melibiose	nd	+
Lactose	+	+
Salicin	−	−
D-Guluconate	+	−
N-Acetylglucosamine	−	−
Succinate, fumarate	+	−
DL-Lactate	−	−
DL-Glycerate	nd	−
Citrate	−	+
Utlization of:		
Aconitate	nd	+
Erythritol	−	−
D-Mannitol	+	+
Glycerol	−	−
γ-Aminobutyrate, sarcosine	nd	−
L-Threonine	+	+
L-Tyrosine	+	+
Putrescine	−	−
D-Sorbitol, DL-malate, α-ketoglutarate, *m*-hydroxybenzoate	−	−
Pigmentation	−	−
Utilization of:		
D-Ribose	−	−
D-Xylose	+	−
L-Arabinose	−	−
L-Rhamnose	−	−

Table 1. Cont'd

Characteristics	H-4	*A. espejiana*
D-Glucose	+	+
Trehalose	−	+
Propionate	+	+
DL-β-Hydroxybutyrate	−	d
Adonitol	−	−
p-Hydroxybenzoate	−	−
L-α-Alanine	+	+
L-Serine	+	+
L-Leucine	+	d
L-Valine	−	−
L-Aspartate	+	+
L-Glutamate	+	−
L-Lysine	−	−
L-Arginine	−	d
L-Ornithine	−	d
L-Citrulline	+	+
L-Proline	+	+
Mol % G+C of DNA	39.8	43–44

nd, not determined; st, straight rod; d, variable; O, oxidative; pol, polar flagellum.

of 38.8 mol% (Tm), oxidatively produced acid from glucose, required seawater for growth. This strain H-4 was identified as a species belonging to the genus *Alteromonas* by Bergey's Manual of Systematic Bacteriology.[3] In the species of genus *Alteromonas*, strain H-4 resembles *A. espejiana* (Table 1). However, it differs from that of *A. espejiana* by the following characteristics: growth at 4°C; requirement for organic growth factors; utilization of D-gluconate, succinate, fumarate, citrate, D-xylose, trehalose, and L-glutamate; and mol% G+C of DNA.

Alginate Lyase Production

The effect of seawater concentration on the growth and alginate lyase production of *Alteromonas* sp. H-4 were investigated in media containing 0.5% casitone and 0.1% sodium alginate (Fig. 2).

The growth levels of *Alteromonas* sp. H-4 were similar in media containing 50, 75, and 90% seawater. However, the highest growth was obtained at 25% seawater (Fig. 2-A). On the contrary, alginate lyase activity was higher in media with increased seawater concentrations of 75 and 90% (Fig 2-B).

The effect of casitone on the growth and alginate lyase production of this bacterium was shown in Fig. 3. With the increase of casitone concentration in media containing 75% seawater and 0.1% sodium alginate, the period of stationary phase

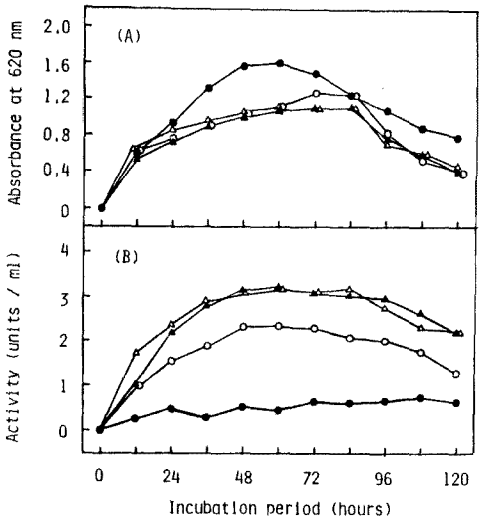

Fig. 2. Effect of seawater concentration on the growth (A) and the alginate layase production (B) of *Alteromonas* sp. H-4.

Seawater concentrations were as follows; 25% (●), 50% (○), 75% (▲), and 90% (△).

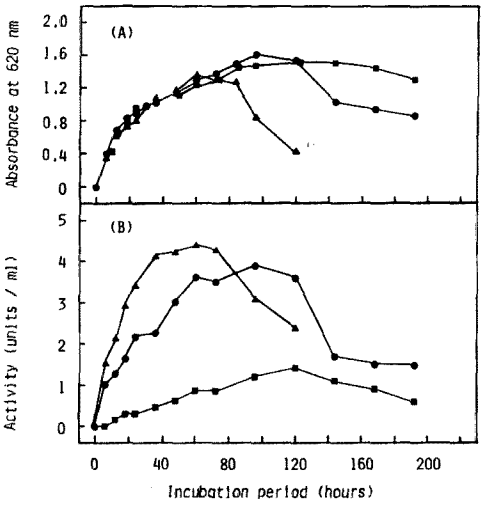

Fig. 3. Effect of the casitone concentration on the growth (A) and the alginate lyase production (B) of *Alteromonas* sp. H-4.

Casitone concentrations were as follows; 0.5% (▲), 0.8% (●), and 1.2% (■).

of *Alteromonas* sp. H-4 was prolonged, and incubation time to give maximum growth yield and release of alginate lyase were also prolonged. Incubation time to give maximum growth (Fig. 3-A) and the enzme activity (Fig. 3-B) in media

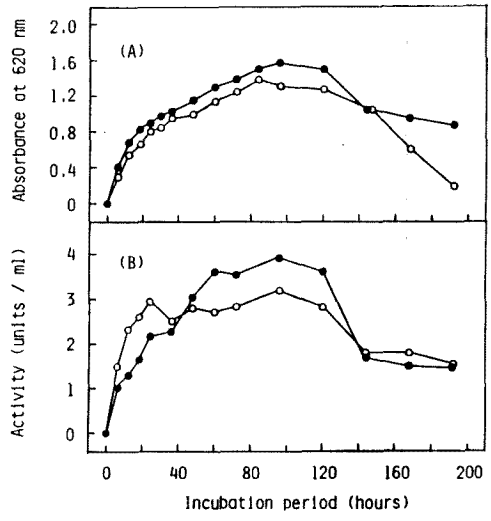

Fig. 4. Effect of sodium alginate on the growth (A) and the alginate lyase production (B) of *Alteromonas* sp. H-4.

Strain H-4 was cultured on media containing 0.1% sodium alginate (●) and without sodium alginate (○).

containing 0.5, 0.8, and 1.2% casitone were 72, 96, and 120 h, respectively. Release of enzyme decreased with an increasing concentration of casitone. When *Alteromonas* sp. H-4 was incubated in medium containing 0.5% casitone. the maximum enzyme activity was obtained only after 72 h incubation but rapidly decreased thereafter.

Changes in growth and alginate lyase activity in media containing 0.1% sodium alginate and without sodium alginate is shown in Fig. 4. On a medium without sodium alginate, alginate lyase was produced by *Alteromonas* sp. H-4. However, in media containing 0.1% sodium alginate, the release of lyase slightly increased as compared with that of without sodium alginate, but the difference of activity between culture with sodium alginate and without sodium alginate was only one unit/ml (Fig. 4-B).

The time course of alginate lyase production by *Alteromonas* sp. H-4 in media containing 0.8% casitone, 0.1% sodium alginate, and 75% seawater is shown in Fig. 5. The enzyme was produced from exponential phase and was on its maximum during stationary phase (96 h), and the time course of alginate lyase production and growth of *Alteromonas* sp. were almost parallel.

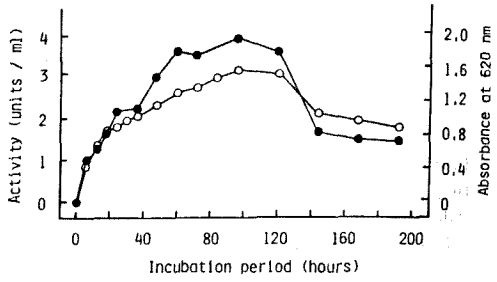

Fig. 5. Time course of the growth and the alginate lyase production of *Alteromonas* sp. H-4.

Strain H-4 was cultured in a medium containing 75% seawater, 0.8% casitone, and 0.1% sodium alginate adjusted to pH 7.5.

●: Alginate lyase activity.

○: Growth of strain H-4.

Discussion

Ando and Inoue[7,8] isolated *Vibrio*-like bacterium from decaying Rishiri-kombu. They reported that the bacterium degrade alginate, and produced holes on small thalli of Rishiri-kombu. We isolated a bacterium from decaying Rishiri-kombu, so-called "*Anaaki-sho*". The strain H-4 completely degraded thallus of kombu for 1 week. We speculated that "*Anaaki-sho*" on Rishiri-kombu is caused by the multiplication of alginolytic bacteria including *Vibrio* sp. and the isolated bacterium.

Strain H-4 from decayed thalli of *L. japonica* var. *ochotensis* closely resembles *A. macleodii*, *A. haloplanktis*, and *A. espejiana* described by Bergey's Manual[3] in biochemical and morphological characters. Of these 3 species, strain H-4 was closest to *A. espejinan* with respect to degrading alginate but were different from each other in some characteristics. Moreover, the determined DNA base composition of strain H-4 was lower (39.8 mol%) than that of *A. espejiana* (43–44 mol%). These results suggest that strain H-4 could not be identified to any species in the genus *Alteromonas*.

Alginate lyases appeared to be present in culture medium during exponential phase of *Alteromonas* sp. H-4, and the change of release of alginate lyase almost corresponded with the growth curve of *Alteromonas* sp. H-4 in all culture conditions tested.

The release of alginate lyase by *Alteromonas* sp. H-4 was highest in media with high concentration of seawater (more than 75%), and with the decrease of seawater concentration in media,

a corresponding decrease in release of alginate lyase was observed. Ions such as Na^+, K^+, and Mg^{2+}, main component of seawater, may protect the activity of alginate lyase. This perhaps explains the increase in alginate lyase activity at higher seawater concentrations and the decrease at lower concentrations.

Alteromonas sp. H-4 produced alginate lyase in media without sodium alginate, and Quatrano & Doubet[9] and Kitamikado *et al.*[10] also reported existence of such alginate lyase producing bacteria. The result indicated that the alginate lyase of *Alteromonas* sp. H-4 might be constitutive enzyme. We are presently investigating the purification and characterization of the enzyme of this strain.

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