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Purification and Morphology of the Flagella of *Vibrio alginolyticus*

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Abstract

The flagella of a marine isolate, *Vibrio alginolyticus* strain 9K-506, were purified by ammonium sulfate precipitation and DEAE-cellulose column chromatography. The flagellar preparations were observed by electron microscope after negatively stained with phosphotungstic acid (PTA).

This organism showed a mixed polar and peritrichous flagellation when grown on agar plates, whereas it formed only a polar monotrichous flagellum in liquid medium. The width of the peritrichous flagellum of the test strain was averaged at 13 nm and that of the polar flagellum with sheath was about 27 nm.

Some marine bacteria, including *Vibrio parahaemolyticus,* possess two kinds of flagella depending on the culture conditions. *Vibrio alginolyticus* is regarded as a biotype of *Vibrio parahaemolyticus* but it is a more ubiquitous bacterium in the coastal region. These bacteria have been assigned to a redefined genus, *Beneckea,* by BAUMANN et al.[1], whereas they are placed in the genus *Vibrio* in the 8th edition of Bergey's manual[2]. Morphological studies of the flagellation of *V. parahaemolyticus* show that this bacterium has a mixed polar (M-flagellum) and peritrichous (L-flagella) flagellation when grown on solid medium, while it has only a polar flagellum when grown in liquid medium[3,4].

The first requirements in the characterization of flagella are the separation and purification properties. Several workers have developed purification procedures for flagella of various bacteria[5,6,7,8]. During the study of flagella of *V. parahaemolyticus,* MIWATANI et al.[9] reported that the peritrichous and monotrichous flagella of this organism were purified separately by preparative zone electrophoresis.

The authors utilized a combination of ammonium sulfate precipitation and DEAE-cellulose column chromatography to purify the flagella from *V. alginolyticus* strain 9K–506. The flagellar preparations obtained by these methods were observed by electron microscope after negative staining with phosphotungstic acid.

Materials and Methods

Bacterial strain and growth media

Vibrio alginolyticus strain 9K–506 isolated from Kinko Bay, Japan was used throughout this work.

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Table 1. Some Comparative Properties of *Vibrio* Species.

Test	Test strain 9K–506	*Vibrio alginolyticus* NIH 154–78	NIH 155–78	*Vibrio parahaemolyticus** 03: K6	05: K15
Colony on BTB teepol	Yellow	Yellow	Yellow	Green	Green
Swarming on agar	++	++	++	−	−
Hugh & Leifson test	F	F	F	F	F
Indole production	+	+	−	+	+
Nitrate reduction	+	+	+	+	+
Gelatin liquefaction	+	+	+	+	+
M. R. test	+	−	+	+	+
V. P. test	+w	+	+w	−	−
Citrate utilization	+	+	+	−	+w
Acid production					
Glucose	+	+	+	+	+
Lactose	+	−	−	−	−
Sucrose	+	+	+	−	−
Growth with					
0% NaCl	−	−	−	−	−
3% NaCl	+++	+++	+++	+++	+++
7% NaCl	++	++	++	+	+
10% NaCl	+w	+	+	−	−

* Authentic strains of *Vibrio* were indebted to Dr. K. TAKAGI at Hokkaido University.
F: fermentative, +w: positive weakly.

A brief characterization of the test strain is shown in Table 1. This strain is positive in the VP test, utilizes citrate as the sole carbon source and ferments glucose, lactose and sucrose.

Bacteria were grown aerobically in liquid or on solid media of modified ZoBell 2216 E at 25 C for 18–20 hours.

Separation of flagella from cells

Cells grown on agar media (50 plates) or in liquid medium (2 *l*) were harvested and then suspended in 30 m*l* of 0.1 M Tris-HCl buffer (pH 7.6). Flagella were removed from cells by treatment for 20 sec with an Ultra-Turrax (IKA-WERK TP18–10). The deflagellated bacteria were removed from the mixture by centrifugation at 10,000 g for 20 min. The precipitated cells were resuspended in 0.1 M Tris buffer and centrifugation was repeated two more times. The pooled supernatant solution was centrifuged at 15,000 g for 20 min to remove the remaining bacterial cells.

Precipitation of flagella with ammonium sulfate

The flagella were precipitated by centrifugation at 10,000 g for 20 min after ammonium sulfate was added to the supernatant solution at 80% saturation. They

were resuspended in 0.1 M Tris buffer and dialysed against 0.1 M Tris buffer at 4 C overnight.

DEAE-cellulose column chromatography

The flagella samples were applied to a DEAE-cellulose column (1.5×25.0 cm) equilibrated with 0.1 M Tris buffer (pH 7.6). The flagella thus adsorbed on the DEAE-cellulose were eluted with a linear gradient of 0–0.5 M NaCl in 0.1 M Tris buffer. Fractions (7 m*l*) were collected and the optical density of each fraction was measured at 280 nm. The fractions containing UV absorbing substances were combined and concentrated by addition of ammonium sulfate. The flagella pellets were then resuspended and dialysed against 0.1 M Tris buffer (pH 7.6).

Chemical analysis

Protein concentrations were determined by the method of LOWRY et al.[10] with bovine serum albumin used as the standard.

Total carbohydrate was measured as glucose equivalents by the anthrone method[11] or the phenol sulfuric acid method[12].

Amino sugar contents were estimated by Morgan-Elson method[13] and Elson-Morgan method of REISSIG[14].

Electron microscope

Copper grids (150 mesh) were coated with 0.7% collodion in amylacetate and carbon stabilized. Grids were inverted on drops of bacterial cells or flagella suspension for 20–30 sec and then 1% PTA solution in ammonium acetate for 15–20 sec. After the prescribed time had passed, the excess solution was blotted with filter paper. Samples thus prepared were observed with a Hitachi H–300 electron microscope at 75 kV.

UV absorbance

UV absorbance of cells and flagella suspensions in 0.1 M Tris buffer was determined by a Shimadzu UV–200 S Double Beam Spectrophotometer.

Results and Discussion

Formation of flagella in liquid or on solid media

The electron micrographs in Fig. 1A and B show bacterial cells with flagella grown in liquid and on solid media, respectively. On cultivation in liquid medium at 25 C for 18 h, most cells formed polar monotrichous flagella and the cell forms were short rods when compared with those grown on solid media. When grown on solid media at 25 C for 18 h, the bacteria were long rods with single polar sheathed and many peritrichous unsheathed flagella. The flagellation of test organism was almost the

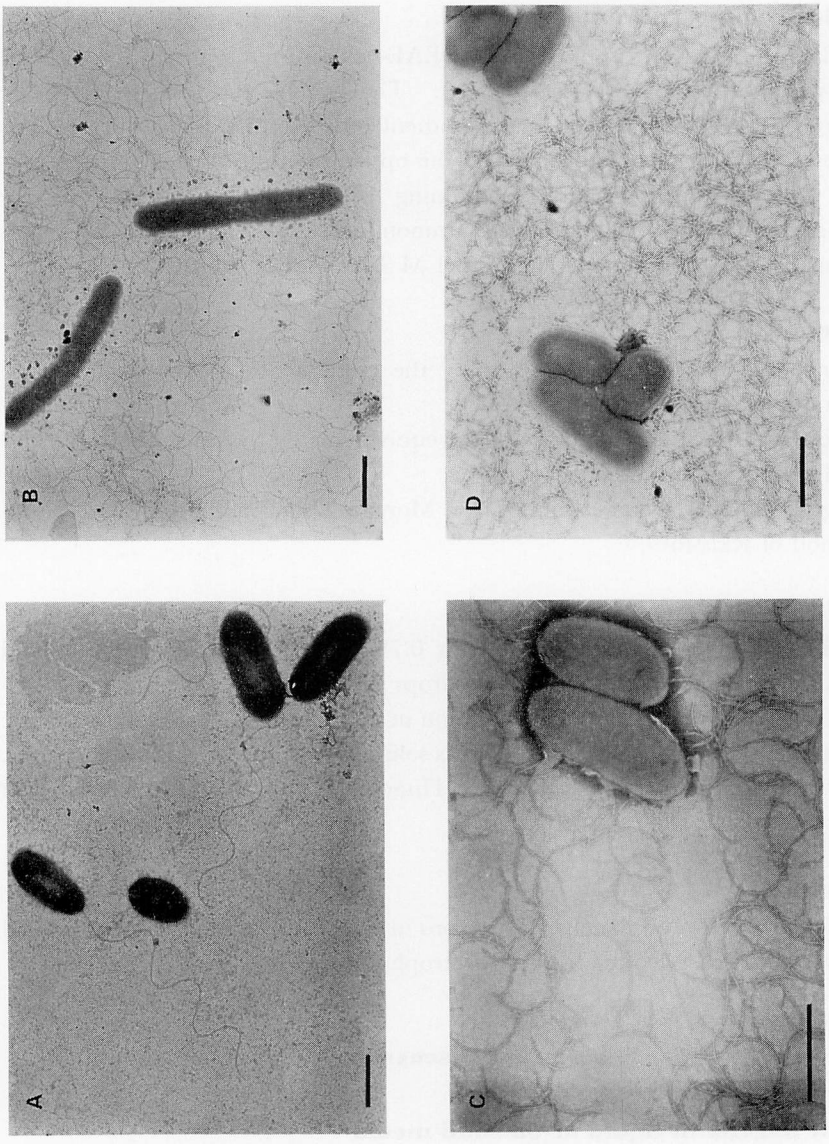

Fig. 1. Electron Micrographs of Strain 9K-506 with Negative Staining.
A; intact cells grown in liquid medium,
B; intact cells grown on solid medium,
C; Turrax treated cells grown in liquid medium,
D; Turrax treated cells grown on solid medium. Bars represent 1.0 μm.

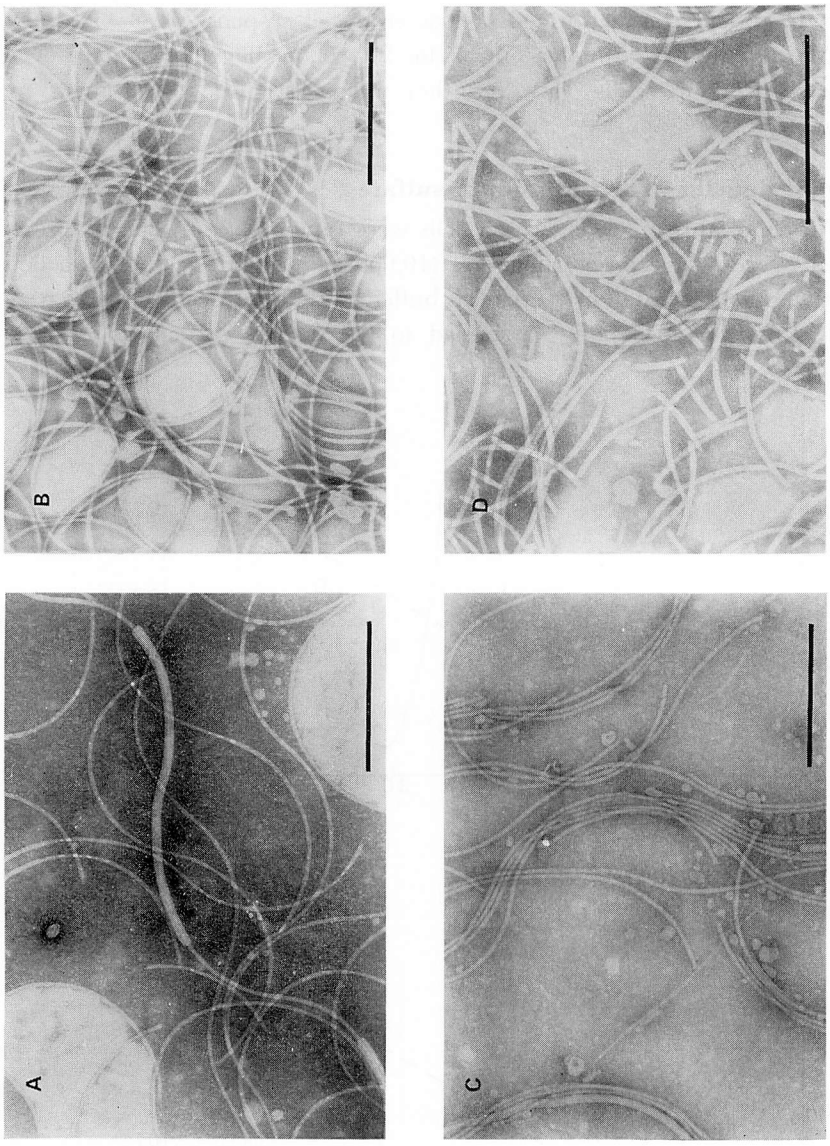

Fig. 2. Electron Micrographs of Flagellar Preparations.
A; Peak Y fractions from DEAE-cellulose column chromatography of flagella obtained on soild culture,
B; ammonium sulfate precipitation of Peak Y derived from solid culture,
C; ammonium sulfate precipitation of Peak C derived from liquid culture,
D; dialysis after ammonium sulfate precipitation of Peak Y derived from solid culture. Bars represent 500 nm.

same as *V. parahaemolyticus* and a marine *Vibrio* B–1 reported previously[15].

Separation of flagella from cells by the Turrax treatment

The Turrax treatment was performed on bacterial cell suspensions of high concentration. After treatment with the Turrax for 20 sec the flagella were removed almost completely from the cells. However, they were shortened to 1–3 curves in length as shown in Fig. 1C and D.

Precipitation of flagella with ammonium sulfate

After the Turrax treatment, deflagellated cells were removed from the mixture of cells and flagella by low speed centrifugation (10,000 g). Then the flagella pellets were recovered and concentrated in 0.1 M Tris buffer by the low speed centrifugation (10,000 g) after ammonium sulfate was added to the flagella suspension at 80% saturation.

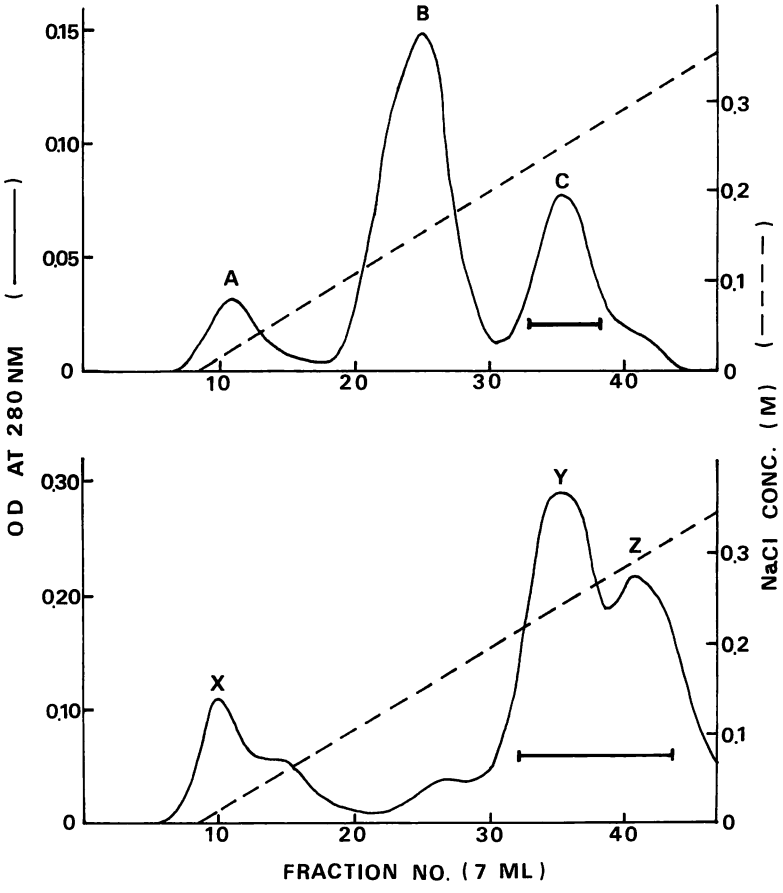

Fig. 3. DEAE-cellulose Column Chromatography of Flagella Obtained from Liquid Culture (Upper) and Solid Culture (Lower).

DEAE-cellulose column chromatography

The flagella suspensions in 0.1 M Tris buffer were further purified using DEAE-cellulose column chromatography. Fig. 3 shows elution profiles of flagellar fractions from DEAE-cellulose column chromatography. Three UV-absorbance peaks were obtained. Peak A (in the case of liquid medium) or Peak X (solid medium) contained bacterial cells. Electron microscopy showed that the other Peaks (Peaks B, Y, C and Z) eluted at NaCl concentrations higher than 0.2 M contained flagella mainly. However it was found that Peak B consisted of a mixture of flagella and membraneous materials in the case of liquid culture. These membraneous materials were considered to be derived from the sheaths of polar flagella. With the DEAE-cellulose column chromatography used in this experiment, both of the polar and peritrichous flagella were eluted at almost the same concentration of NaCl (0.23 M).

The plates (Fig. 2) show electron micrographs of flagellar preparations after ammonium sulfate precipitation and dialysis of fractions from DEAE-cellulose column chromatography. The width of the peritrichous flagellum is approximately 13 nm and that of the polar flagellum with sheath is 27 nm.

MIWATANI et al.[9] reported that four protein fractions were obtained during electrophoresis of flagella preparations of *V. parahaemolyticus* and that three of these were contaminated with spherical bodies. The flagellar fractions obtained in this experiment had few of the spherical bodies or membraneous materials. This may be due to the methods of mechanical removal of flagella from the cells.

Table 2. Chemical Analysis of Flagellar Preparations.

Preparations	Contents (mg/l of culture)				
	Protein (Lowry method)	Total carbohydrate (Anthrone)	Total carbohydrate (Phenol)	Amino sugar (Elson-Morgan)	Amino sugar (Morgan-Elson)
Liquid culture					
Cell pellets	153 (100%)	6.5 (4.2%)	21.0 (13.7%)	9.5 (6.2%)	0.7 (0.4%)
Flagella					
before column	28 (100)	0.40 (1.4)	0.88 (3.1)	ND	ND
after column	NE	0.04	0.16	ND	ND
Solid culture					
Cell pellets	323 (100)	21.0 (6.5)	43.0 (13.3)	19.0 (5.9)	1.8 (0.6)
Flagella					
before column	73 (100)	0.9 (1.2)	1.6 (2.2)	ND	ND
after column	75 (100)	0.2 (0.2)	0.3 (0.4)	ND	ND

NE: not examined, ND: not detected, column: DEAE-cellulose column chromatography.

Chemical analysis of flagellar fractions

Table 2 shows the contents of protein, reducing sugar and amino sugar in flagellar fractions. The flagellar fractions were found to consist of protein as the main com-

ponent. The concentrations of carbohydrate were relatively low and amino sugar was not detected at all. UV absorbance spectra showed that flagellar fractions have a maximum absorbance at 280 nm and absorbance at 250 nm was lower compared with that of cell suspensions (Fig. 4). These results support the belief that the flagella are composed of protein subunits.

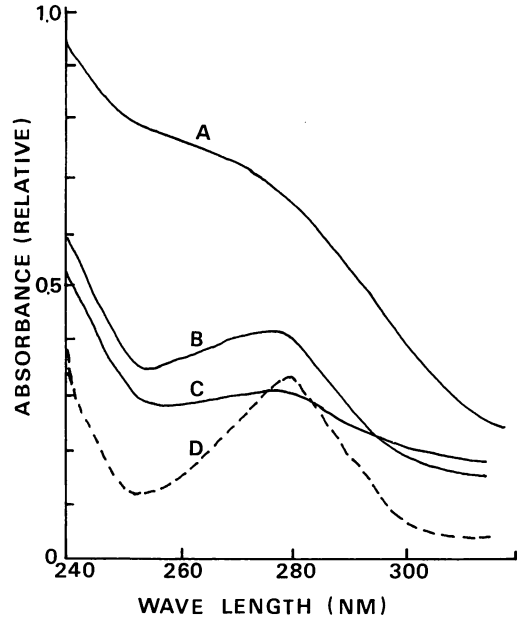

Fig. 4. Absorption Spectra of Cells and Flagella Preparations.
A; cell suspension,
B; Peak Y fraction derived from solid culture,
C; Peak C fraction derived from liquid culture,
D; bovine serum albumin solution.

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