Variation in Flagellar Morphology of *Vibrio parahaemolyticus* and *Vibrio alginolyticus* in Different Cultural Conditions

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Three strains, namely the K+ and K- (Kanagawa positive and negative) strains of *V. parahaemolyticus* and a strain of *V. alginolyticus* were used to study the variation of flagellar morphology in different cultural conditions. All three strains showed M-(monotrichous) flagella in liquid media in different studies. But on solid media, the flagellar morphology varied depending on the type of media, levels of pH, NaCl concentrations, growth temperatures and other cultural conditions. Examination of flagella during the growth period of the strains revealed that *V. alginolyticus* and K+ strain of *V. parahaemolyticus* first showed PM-flagella and then changed to P-(peritrichous) flagella, whereas K- strain showed P-flagella throughout the growth period. The strains developed M-flagella when they were grown on TS3%NaCl medium containing gelatin (7%) and agar (0.75%) as solidifying agents. However, the same strains showed either mixed type or P-flagella when they were grown on ZoBell 2216E medium made with either different concentrations of agar or carrageenan. It was observed that the relation between swarming and P-flagellation depended on cultural conditions. The results obtained in the present study indicate the need for standardization of cultural conditions when establishing flagellar morphology.

The flagellar morphology plays an important role in bacterial classification. Hence, the flagellar morphology should be established in different cultural conditions before it is used in the taxonomy of bacteria. SAKAZAKI *et al.*¹⁾ reported that *V. parahaemolyticus* had a single polar flagellum. MIWATANI *et al.*²⁾ observed sheathed polar flagellum of *V. parahaemolyticus*. BAUMANN *et al.*^{3,4)} ALLEN and BAUMANN,⁵⁾ NAKAMURA *et al.*⁶⁾ and SAKATA *et al.*⁷⁾ reported that *V. parahaemolyticus* or *V. alginolyticus* produced a polar monotrichous sheathed flagellum in a liquid medium and on a solid medium they produced in addition to single polar sheathed flagellum, unsheathed peritrichous flagella. SHINODA and OKAMOTO⁸⁾ studied the formation and function of lateral flagella in *V. parahaemolyticus*.

The study conducted so far on flagellar morphology of *V. parahaemolyticus* and *V. alginolyticus* is limited in the sense that the flagellar morphology has been studied on a certain medium at a definite pH and temperature, and there is no report on flagellar morphology in different cultural conditions. In view of the above, the present study was undertaken to study the variation of flagellar morphology in different cultural conditions.

Swarming in relation to flagellar morphology was studied. The Kanagawa positive and negative strains of *V. parahaemolyticus* were compared from the point of variability in flagellar morphology.

Materials and Methods

Strains

Three strains; a Kanagawa positive (VPHK-6,03: K6) and a Kanagawa negative (VPHK-6,06: K46) strains of *V. parahaemolyticus*, and a strain of *V. alginolyticus* (VAF2. NIH 155-78) were used in the study. The source and maintenance of the strains have been described by ANAND *et al.*⁹⁾

Inocula

A 24 h culture of Trypticase Soy Broth supplemented with 3% NaCl was used for inoculation in different cultural studies. Controls were run in all experiments.

Cultural Conditions

Media

For studying flagellar morphology in different

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media, the following media were used: a) ZoBell 2261E agar and broth (the composition of which has been described in the previous paper⁹) b) Trypticase Soy agar and broth (Baltimore Biological Labs.) supplemented with 3% NaCl c) BTB teepol agar (Eiken Chemical Co.) d) TCBS agar (Eiken) e) Vibrio agar (Nissui Chemical Co.). In addition, for making semisolid medium, ZoBell 2216E medium with 0.6% agar was used.

pH, NaCl and Temperature

For examining flagellar morphology of strains grown in different pH (6, 7.5 and 9), NaCl concentrations (1, 3, 5 and 7%) and temperatures (18, 21, 25, 37 and 42°C), ZoBell 2216E agar and broth media were used. The flagellar were examined after incubating the streaked plates and broth tubes at 25 and 37°C for 18 h.

Artificial Sea Water (ASW)

To study the influence of ASW, ZoBell 2216E agar and Trypticase Soy agar with 3% NaCl media were used. In addition, the same ZoBell medium with 3% NaCl instead of ASW and TSA medium with ASW instead of 3% NaCl were used. The flagella were examined after incubating the streaked plates for 18 h at 25 and 37°C.

Incubation Time

Morphology of flagella at different incubation times were examined using ZoBell 2216E agar medium. The flagella were examined after incubating the streaked plates for 6, 12, 18, 24 and 38 h at 37°C.

Effect of Solidifying Agents

Flagellar morphology of cells grown on ZoBell 2216E medium made with 0.5, 1.0, 1.5 and 2.0% agar was observed after 18 h of incubation at 25 and 37°C. The viscosity of ZoBell 2216E medium made with 1.5% agar was increased by adding 1% carboxymethyl cellulose (Na salt, Wako) and flagella were examined after 18 h of incubation at 25 and 37°C. Gelatin (7%) in combination with agar (0.75%) was used as solidifying agent in

TSB 3% NaCl medium to see if it exerts any influence on flagellar morphology of the strains. The flagella were examined after incubating plates for 24 h at 15 and 21°C.

ZoBell 2216E solid medium was made with 1.5% carrageenan (Matsumoto Co.) as solidifying agent and the strains were examined for flagella after incubating the streaked plates for 18 h at 25 and 37°C.

Swarming

Streak plate method was used to observe swarming on solid media in different cultural conditions.

Examination of Flagella

The flagella were examined by means of an electron microscope. Bacterial samples were placed on collodion coated, carbon coated 150 mesh copper grids. The grids were stained negatively with 1% potassium phosphotungstate (pH 7.0). The specimens were observed in a Hitachi H-300 electron microscope at an accelerating voltage of 75 KV.

Results

Flagellar Morphology in Different Cultural Conditions

Media

As seen in Table 1, all strains showed peritrichous (P) flagella at 25 and 37°C on solid media of ZoBell 2216E (Fig. A) and TSA 3% NaCl, while in liquid media they showed monotrichous (M) flagella at both the temperatures. In the case of Vibrio agar, BTB Teepol and TCBS solid media (Fig. B and C), all the strains showed M-flagella at both 25 and 37°C. The amount of P-flagella as observed on grids in the electron microscope was more when ZoBell 2216E medium was used than when TSA 3% NaCl medium was used. On semi solid medium of ZoBell 2216E with 0.6% agar, the strains showed a mixed monotrichous and peritrichous flagellation; the Kanagawa negative strain show-

Table 1. Flagellar morphology of strains grown in different media

Medium Incubation temp. °C	BTB agar		TCBS agar		Vibrio agar		ZoBell 2216E				TS+3%NaCl			
							agar		broth		agar		broth	
	25	37	25	37	25	37	25	37	25	37	25	37	25	37
<i>Strain</i>														
VAF2	M* ¹	M	M	M	M	M	P* ²	P	M	M	P	P	M	M
VPHK6	M	M	M	M	M	M	P	P	M	M	P	P	M	M
VPFK46	M	M	M	M	M	M	P	P	M	M	P	P	M	M

*¹ Monotrichous flagella.

*² Peritrichous flagella.

ed PM-flagella (cells with P-flagella more than with M-flagella), whereas the other two strains showed MP-flagella (cells with M-flagella more than with P-flagella).

pH, NaCl, ASW and Temperature

In liquid medium, all cultures showed M-flagella at all pH (6, 7.5 and 9), NaCl concentrations (1, 3, 5 and 7%) and temperatures (18, 21, 25, 37 and 42°C) used in the study. On solid medium, the flagellar morphology varied. In pH studies, at 25°C, the flagellation of *V. parahaemolyticus* strains (both K+ and K-) changed from M-type at pH 6 to P-type at pH 7.5 and then to MP-type at pH 9. Whereas *V. alginolyticus* showed MP-flagella at pH 6, P-flagella at pH 7.5 and PM-flagella at pH 9 (Table 2). At 37°C, *V. alginolyticus* and K+ strain of *V. parahaemolyticus* showed M, P and again M-flagella at 6, 7.5 and 9 respectively (Fig. D). The K- strain showed M-flagella at pH 6 and P-flagella at pH 7.5 and 9. The study with NaCl concentrations at 25 and 37°C (Table 3) indicated that while all the strains developed P-flagella at 3% NaCl and M-flagella at 7% NaCl,

the flagellar type was different at 1 and 5% NaCl concentrations in all the three strains. At 25°C, *V. alginolyticus* showed P and MP-flagella respectively at 1 and 5% NaCl concentrations; whereas the strains of *V. parahaemolyticus* showed PM and MP-flagella at 1 and 5% NaCl concentrations, respectively. At 37°C, *V. alginolyticus* developed flagella of M-type at 1% (Fig. E) and PM-type at 5% NaCl concentrations. While the K+ strain of *V. parahaemolyticus* showed PM-type at 1% and MP-type at 5%, K- strain showed M and MP-flagella at 1 and 5% NaCl concentrations, respectively.

When the strains were cultivated on ZoBell 2216E medium made with either ASW or 3% NaCl and flagella were examined, there was no difference in flagellar type. The same result was obtained with TSA medium made with either ASW or 3% NaCl. This observation showed that mineral components of ASW besides NaCl did not exert any influence on variation in flagellar type.

When the strains were grown at different temperatures and flagella were examined (Table 4), all the three strains showed P-flagella at 25 and 37°C, but at 42°C they showed M-flagella. At 18 and 21°C, *V. alginolyticus* showed MP-flagella; whereas the strains of *V. parahaemolyticus* showed PM-flagella.

Table 2. Flagellar morphology of strains grown at different pH on solid medium

Incubation temp. °C pH	25			37		
	6.0	7.5	9.0	6.0	7.5	9.0
Strain						
VAF2	MP*1	P	PM*2	M	P	M
VPHK6	M	P	MP	M	P	M
VPFK46	M	P	MP	M	P	P

*1 Cells with M-flagella more than cells with P-flagella.

*2 Cells with P-flagella more than cells with M-flagella.

Table 3. Flagellar morphology of strains grown on solid medium at different NaCl concentrations

Incubation temp. °C NaCl Conc. %	25				37			
	1	3	5	7	1	3	5	7
Strain								
VAF2	P	P	MP	M	M	P	PM	M
VPHK6	PM	P	PM	M	PM	P	PM	M
VPFK46	PM	P	MP	M	M	P	MP	M

Table 4. Flagellar morphology of strains grown at different temperatures on solid medium

Temp. °C	18	21	25	37	42
Strain					
VAF2	MP	MP	P	P	M
VPHK6	PM	PM	P	P	M
VPFK46	PM	PM	P	P	M

Incubation Time

The flagellar morphology of the strains at different incubation times (6, 12, 18, 24 and 36 h), grown at 37°C on ZoBell 2216E agar, was examined to see if there was any change in flagellar type during their growth period. *V. alginolyticus* first showed PM-flagella in 6 h culture and then changed to P-flagella at other incubation times (Fig. I and J). The K+ strain of *V. parahaemolyticus* showed PM-flagella in 6 and 12 h cultures and after that time it changed to P-flagella. The K- strain showed P-flagella throughout the growth period (Fig. F, G and H). In 38 h culture though the flagella were P-type in all the strains, many cells were found to be deflagellated (Fig. K).

Effect of Solidifying Agents

When agar was used as solidifying agent in ZoBell 2216E medium at different concentrations (0.5, 1, 1.5 and 2%), the flagellar type of the strains varied with respect to the concentrations used (Table 5). It also slightly depended on the growth temperature of the strains. At 25°C, all the three strains showed P-flagella at 1 and 1.5% concentrations. At 0.5 and 2% concentrations, *V. alginolyti-*

Fig. A to M. Electron micrographs. Cells were negatively stained.

Fig. A. K+ strain of *V. parahaemolyticus* (VPHk6), grown on ZoBell 2216E solid medium at 25°C. $\times 3500$

Fig. B. VPHk6, grown on BTB Teepol solid medium at 25°C. $\times 4900$

Fig. C. VPHk6, grown on TCBS solid medium at 25°C. $\times 350$

Fig. D. VPHk6, grown on ZoBell 2216E(pH 6) solid medium at 37°C. $\times 7000$

Fig. E. *V. alginolyticus*, grown on ZoBell 2216E (1% NaCl) solid medium at 37°C. $\times 7000$

Fig. F. K+ strain of *V. parahaemolyticus* (VPFk46), 12 h incubation, grown on ZoBell 2216E agar at 37°C. $\times 4900$

Fig. G. VPFk46, 6 h incubation, grown on ZoBell 2216E agar at 37°C. $\times 7000$

Fig. H. VPFk46, 18 h incubation, grown on ZoBell 2216E agar at 37°C. $\times 7000$

Fig. I. *V. alginolyticus* (VAF2), 12 h incubation, grown on ZoBell 2216E agar at 37°C. 7000

Fig. J. VAF2, 38 h incubation, grown on ZoBell 2216E agar at 37°C. $\times 4900$

Fig. K. VAF2, 38 h incubation, grown on ZoBell 2216E agar at 25°C. $\times 3500$

Fig. L. VAF2, grown on ZoBell 2216E+1.5% carrageenan solid medium at 37°C. $\times 7000$

Fig. M. VPHK6, grown on TSB 3% NaCl+7% gelatin+0.75% agar solid medium at 21°C. $\times 4900$

Table 5. Flagellar morphology of strains grown at different agar concentrations

Incubation temp. °C Agar conc. %	25				37			
	0.5	1.0	1.5	2.0	0.5	1.0	1.5	2.0
Strain								
VAF2	MP	P	P	MP	MP	M	P	P
VPHK6	MP	P	P	PM	MP	MP	P	MP
VPFK46	PM	P	P	MP	PM	P	P	MP

cus showed MP-flagella, K+ strain of *V. parahaemolyticus* showed MP and PM-flagella, and K- strain showed PM and MP-flagella, respectively. At 37°C, the flagellar type of *V. alginolyticus*, K+ and K- strains of *V. parahaemolyticus* was respectively MP, M, P and P; MP, MP, P and MP; PM, P, P and MP at 0.5, 1, 1.5 and 2% agar concentrations. When viscosity of the ZoBell 2216E medium made with 1.5% agar was increased by adding 1% carboxymethyl cellulose, all the three strains developed P-flagella at both 25 and 37°C. The amount of P-flagella was more when observed visually on the grids in the electron microscope.

When gelatin (7%) alone was used, it was liquified by the strains during incubation. Hence, gelatin (7%) in combination with agar (0.75%) was used as solidifying agent in TSB 3% NaCl medium. Surprisingly, all strains showed M-flagella at both 15 and 21°C (Fig. M).

When carrageenan at 1.5% concentration was used as solidifying agent instead of agar in ZoBell 2216E medium, all the three strains showed P-flagella (Fig. L).

Swarming

When the strains were grown on ZoBell 2216E medium at 25 and 37°C, *V. alginolyticus* swarmed rapidly, K- strain of *V. parahaemolyticus* swarmed slowly and K+ strain did not swarm at both the temperatures. The examination of flagella showed that they were all peritrichous. The strains of *V. parahaemolyticus* did not swarm on TSA 3% NaCl plates at both 25 and 37°C but *V. alginolyticus* swarmed only at 25°C and not at 37°C. The flagella were P-type in all the strains. It was observed that all the three strains did not swarm at 18 and 42°C, and the flagella of the strains were either mixed MP or PM and M-type at the respective growth temperatures. It was also observed that while *V. alginolyticus* swarmed on TSA 1% NaCl plates at both 25 and 37°C, K- strain of *V. parahaemolyticus* swarmed only at 25°C but not at 37°C. When flagella were examined *V. alginolyticus* showed peritrichous at 25°C and monotrichous at 37°C while K- strain of *V. parahaemolyticus* showed mixed PM-type at 25°C and M-type at 37°C.

Discussion

BAUMANN *et al.*^{3,4)} and ALLEN and BAUMANN⁵⁾ observed that the strains of *V. parahaemolyticus* and *V. alginolyticus* showed P-flagella on solid medium and M-flagella in liquid medium. The medium used in their studies was either yeast extract of Difco marine agar medium. YABUCHI *et al.*¹⁰⁾ found that the cells of *V. parahaemolyticus* had both polar and peritrichous flagella when grown on modified MOF agar after 16 h incubation at 20°C. In the present study, the flagellar morphology of the strains depended on the type of medium used (Table 1). This observation calls for a need for specification of a medium for establishing flagellar morphology if shape and arrangement of flagella is considered as a criterion in the classification of bacteria. The reason for showing M-flagella on Vibrio agar, BTB Teepol and TCBS solid media, in the present study, may be due to composition of the medium coupled with high pH (>8) used in the medium.

SHINODA and OKAMOTO⁹⁾ observed that on solid medium cells of *V. parahaemolyticus* formed lateral flagella at both 37 and 25°C, although at 37°C they became dissociated from the cells and decomposed in the medium. ULITZUR¹¹⁾ reported that temperatures above 28°C inhibited the formation of peritrichous flagella by *V. alginolyticus*. It was observed in our study that at 18 and 21°C, the strains developed mixed type flagellation; at 42°C, M-flagellation; and at 25 and 37°C P-flagellation although some cells were found to be deflagellated at 37°C.

When flagella were examined at different incubation times, all strains showed P-flagellation at 24 h but at 38 h, though the flagella were P-type, many cells were found to be deflagellated. This observation differs from the result obtained by SHINODA and OKAMOTO⁹⁾ who found that cells of *V. parahaemolyticus* after cultivation at 37°C for 18 h had no lateral flagella, although the amount of lateral flagella was maximum in 6 h culture.

SHINODA and OKAMOTO⁹⁾ also found that when *V. parahaemolyticus* was grown at different agar concentrations, the amount of lateral flagella was maximum at 0.9%, but it decreased at 1.2% and then again slightly increased at 1.5%. They have not conducted study at 2% agar. It was noted in the present study that at 25°C, all the three strains showed P-flagella at 1 and 1.5% agar and mixed type flagellation at 0.5 and 2% agar concentrations (Table 5).

All the three strains showed M-flagella when they

were grown on TS 3%NaCl medium made with gelatin (7%) and agar (0.75%) as solidifying agents. The same three strains showed either mixed type or P-flagellation when they were grown at different agar concentrations (Table 5). This observation showed that gelatin in combination with agar seemed to vary the type of flagellation.

In the study on swarming in relation to flagella, it was observed that the relation between swarming and peritrichous flagellation depended on the cultural conditions. For example, *V. alginolyticus* when grown on ZoBell 2216E solid medium swarmed at 25 and 37°C, and the flagella were peritrichous. But when the same strain was grown on TSA 1% NaCl plates at 25 and 37°C, though they swarmed at both the temperatures, the flagellation was peritrichous at 25°C and monotrichous at 37°C. This result differs from the result of ULITZUR¹¹⁾ who found that temperatures above 28°C inhibited both the formation of peritrichous flagella and swarming in *V. alginolyticus*. It can be said from the observations made in the present study that swarming need not always be related to peritrichous flagellation. In conclusion, the results obtained in the present study indicate the need for standardization of cultural conditions for establishing flagellar morphology.

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