Multiflagellate Variants of *Vibrio anguillarum*

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An ultrastructural examination of six strains of *Vibrio anguillarum* of varying virulence for eels revealed an apparent correlation between pathogenicity and the possession of more than one flagellum. The relationship between *V. anguillarum* surface appendages and virulence is discussed.

INTRODUCTION

Vibrio anguillarum is an important pathogen of marine and estuarine fish, causing the haemorrhagic septicaemia vibriosis (Harbell *et al.*, 1979). Although *V. anguillarum* appears to infect all teleosts (Anderson & Conroy, 1970), vibriosis has been of greatest concern to intensive fish culture industries (Cisar & Fryer, 1969; Novotny, 1978).

A prelude to vibriosis is an initial colonization of the host by *V. anguillarum* with a subsequent penetration of the fish integument. Pathogenic bacteria have been shown to have surface appendages which enable bacteria to locate, adhere to and penetrate mucosal surfaces (Hazelbauer & Parkinson, 1977; Jones, 1977; Doetsch & Sjoblad, 1980). Possible routes by which *V. anguillarum* enters the host have been suggested (Roberts *et al.*, 1973; Hästein & Bergsjö, 1976), but the means by which this organism gains access to the host tissues is unknown.

To assist in understanding the early stages of host colonization, an ultrastructural study of *V. anguillarum* was carried out. By comparing ultrastructural observations with strain virulence, a correlation was demonstrated between ability to cause disease in eels and the possession of more than one flagellum.

METHODS

Bacterial culture. Strains of *V. anguillarum* used in this study (Table 1) were maintained in aqueous skimmed milk (100 g l⁻¹) under liquid nitrogen refrigeration. Bacteria initially cultured on blood agar, which comprised tryptone soy agar (TSA, Oxoid), plus NaCl (15 g l⁻¹) and sterile horse blood (5%, v/v), at 25 °C, were further sub-cultured in tryptone soy broth (TSB, Oxoid).

Bacterial identification. Salient bacteriological and biochemical tests used for the identification of isolates were based on those of Hendrie *et al.* (1971) using the type strain NCMB 6 for comparison.

Determination of virulence. Groups of 15 eels (*Anguilla anguilla*) (mean weight 63.2 g, S.E. 8.5 g) maintained in 50% (v/v) seawater at 22 °C were used to determine the lethal dose-50 (LD₅₀) of strains, calculated by the method of Reed & Meunch (1938). Broth-cultured bacteria were serially diluted (10-fold dilutions) in phosphate-buffered saline (1.05%, w/v, NaCl). Volumes (0.1 ml) of each dilution were injected intraperitoneally into groups of eels and the number of viable bacteria administered was determined by bacterial counts (Miles *et al.*, 1938) on each dilution.

Mortalities were recorded three times daily and the experiment terminated after 7 d. Post mortem, kidney and spleen tissues were aseptically dissected and streaked on to blood agar. Resultant growth was identified to the bacterial genus (see above), and *Vibrio* spp. were identified as *V. anguillarum* by fulfilment of Koch's postulates.

Electron microscopy. Bacteria, placed on Formvar-coated, carbon-stabilized copper grids, were examined by transmission electron microscopy (Philips 300 TEM), after negative staining with aqueous ammonium molybdate or potassium phosphotungstate (10 g l⁻¹, pH 7.0), or shadowing with gold or gold-palladium. Grids were examined at 80 kV and photographed using electron image sheet film (Kodak).

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Table 1. *Sources of Vibrio anguillarum strains used*

Strain	Source	Reference
UNH 569	R. G. Strout, U.S.A.	—
HC 2	J. Rohovec, U.S.A.	—
MS 549	U.S.A.	—
COB 408	F. Baudin-Laurencin, France	Baudin-Laurencin & Tangtrongpiros (1980)
MS 2072	U.S.A.	—
PB 15	Japan	Aoki <i>et al.</i> (1981)
NCMB 6	J. & O. Bagge, Denmark	Bagge & Bagge (1956)

Table 2. *Results of biochemical and bacteriological identification tests for Vibrio anguillarum strains*

Test	UNH 569	HC 2	MS 549	COB 408	MS 2072	PB 15	NCMB 6
Gram stain	—	—	—	—	—	—	—
Motility	+	+	+	+	+	+	+
Oxidase production	+	+	+	+	+	+	+
Catalase production	+	+	+	+	+	+	+
Gas from glucose	—	—	—	—	—	—	—
Glucose fermentation	+	+	+	+	+	+	+
O/129 sensitivity	+	+	+	+	+	+	+
Growth in TSB:							
3% (w/v) NaCl	+	+	+	+	+	+	+
7% (w/v) NaCl	—	—	—	—	—	—	—
at 25 °C	+	+	+	+	+	+	+
at 30 °C	+	+	+	+	+	+	+
at 37 °C	—	+	+	+	+	+	—
at 42 °C	—	—	—	—	—	—	—
Acid from:							
Arabinose	—	+	+	—	—	+	+
Cellobiose	—	+	+	+	+	—	+
Dulcitol	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+
Lactose	—	—	—	—	—	—	—
Maltose	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+
Sorbitol	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+
Indole production	—	—	—	+	+	+	+
Gelatin liquefaction	+	+	+	+	+	+	+
Starch hydrolysis	+	+	+	+	+	+	+
Urease production	—	—	—	—	—	—	—
Methyl red test	—	—	—	—	—	—	—
Haemolysin production	+	+	+	+	+	+	+

RESULTS

Bacteriological and biochemical tests revealed considerable similarity between the strains used in these experiments and the type strain, NCMB 6 (Table 2).

Of six strains of *V. anguillarum* examined, three were found to be virulent (LD_{50} : strain UNH 569, 1.1×10^7 ; strain HC 2, 2.1×10^7 ; strain MS 549, 3.4×10^8), while the remainder had LD_{50} values greater than 10^9 viable bacteria per fish.

By negative staining, bacteria were observed to have a wrinkled outer surface and one, two or three flagella located at the pole of the cell (Fig. 1*a*). It was evident that some strains produced more multiflagellate individuals than others. However, cells with more than one flagellum were only observed in strains designated virulent. Strain HC 2 had the highest incidence of cells with more than one flagellum (5.5% with three, 21.0% with two). Flagella were approximately 30 nm

Fig. 1. Electron micrographs of *V. anguillarum*: (a) negatively stained cell (bar represents 0.5 μm); (b) flagellum shadowed with gold-palladium showing inner core (arrowed) surrounded by an outer sheath (bar represents 0.1 μm).

in diameter and generally three to four times longer than the bacterial cell. Intact flagella had a terminal swelling at the distal terminus, about 40 nm in diameter. Metal-shadowed preparations of damaged flagella revealed a core region (12 nm in diameter) surrounded by an outer sheath (Fig. 1b).

Negative staining and shadowing revealed the occasional presence of pili (7.5 nm diameter) in strains HC 2 and HC 4; however, these observations were rare. For this purpose, ammonium molybdate and gold-palladium were found to give superior resolution to potassium phosphotungstate and gold alone.

DISCUSSION

The protocol employed for infecting eels with *V. anguillarum* was similar to those used by other workers for assessing the pathogenicity of this organism (Evelyn, 1971; Traxler & Li,

1972; Schiewe & Hodgins, 1977). LD₅₀ values obtained here for strains of highest virulence (approximately 10⁷) were considerably higher than the highest value observed in coho salmon (10³: Crosa *et al.*, 1977) and ayu (8.75 bacteria: Jo & Muroga, 1977). Interestingly, strain 775 used by Crosa *et al.* (1977) was found to be of low virulence in eels (Chart, unpublished), suggesting that the eel is a particularly resistant species, which has been reported elsewhere (Larsson & Fänge, 1969).

Vibrio anguillarum has previously been described as possessing a single polar flagellum (Hästein, 1974; Roberts, 1978). However, this report presents evidence that multiflagellate forms do exist and that there was a correlation between virulence and the possession of more than one flagellum. How multiflagellation plays a role in pathogenesis is unclear, and whether these forms occur *in vivo* has yet to be determined. Nevertheless, *V. anguillarum* has been shown to be highly invasive (Chart, unpublished) and possibly additional flagella may be involved in chemotaxis and/or penetration of mucosal surfaces.

Motility of *V. cholerae* has been reported as being essential for chemotactic location and penetration of rabbit intestinal mucus (Jones *et al.*, 1976) and the possession of flagella appears to have some involvement in adherence of bacteria to mucosal surfaces by flagellar adhesin production (Jones *et al.*, 1976; Jones, 1980). Possible involvement of flagella in locating fish mucous membranes and in subsequent attachment has not been investigated. However, it has been demonstrated that water-borne *V. anguillarum* are taken into the gut of marine-acclimatized eels with imbibed water as a consequence of osmoregulation (Chart, unpublished), which may be a prelude to mucosal colonization.

Although some virulent bacteria had more than one flagellum per cell, the general flagellar structure appeared similar. The structure of the *V. anguillarum* flagellum appeared similar to that of *V. metschnikovii* and other *Vibrio* spp. (Glauert *et al.*, 1963; Follet & Gordon, 1963; Ogasawara & Kuno, 1964), which has a central core composed of flagellin, surrounded by a sheath which is continuous with the outer membrane. The terminal swelling observed in *V. anguillarum* appeared to be slightly smaller than that observed in *V. cholerae* (biotype El Tor) (Rowles *et al.*, 1976).

Although two strains of *V. anguillarum* were shown to possess pili, this feature was only observed in relatively few cells and their involvement in virulence could not be determined. Nevertheless, possession of pili has been implicated as a major virulence factor in some human pathogens (Swanson *et al.*, 1971; Jephcott *et al.*, 1971) where attachment to epithelia plays an important role in pathogenesis.

Although the test strains were not identified by DNA hybridization, biochemical data for the strains used strongly suggest that the test strains were *Vibrio anguillarum*.

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