

Influences of temperature, salinity and starvation on the motility and chemotactic response of *Vibrio anguillarum*

Marianne H. Larsen,¹ Nicholas Blackburn,² Jens L. Larsen¹ and John E. Olsen¹

Correspondence
John E. Olsen
jeo@kvl.dk

¹Department of Veterinary Microbiology, The Royal Veterinary and Agricultural University, Stigboejlen 4, DK-1870 Frederiksberg C, Denmark

²Marine Biological Laboratory, University of Copenhagen, Strandpromenaden 5, 3000 Helsingør, Denmark

The role of growth factors for the motility and chemotaxis of the fish pathogen *Vibrio anguillarum* was determined. Cells of *V. anguillarum* were chemotactic to serine in the temperature range 5–25 °C and in 0.8–2.7% NaCl. The chemotactic response was significantly higher at 25 °C than at 5 or 15 °C. Growth in medium with 1.5% NaCl gave a higher response than growth with 3% NaCl; when the salinity of the chemotaxis buffer was raised, the chemotactic response was reduced. The role of starvation was also studied; *V. anguillarum* showed a high chemotactic response after starvation for 2 and 8 days. Motility and chemotaxis are important virulence factors for this bacterium. Not only was the ability to perform chemotactic motility maintained after starvation, but also it was shown that starvation does not interfere with the ability of the organism to cause infection in rainbow trout after a bath challenge. The swimming speed was reduced at lower temperatures. Within the range of salinity and starvation studied, the motile cells swam with the same velocity, indicating that *V. anguillarum* under all the examined conditions has a functional flagellum and rotates it with constant speed. Phenamil, a specific inhibitor of Na⁺-driven flagella, reduced the motility of both starved and non-starved cells of *V. anguillarum* indicating that, in both cases, a Na⁺ motive force drives the flagellum.

Received 31 March 2003
Revised 23 December 2003
Accepted 7 January 2004

INTRODUCTION

Vibrio anguillarum causes vibriosis in a variety of fish species in marine or brackish environments at water temperatures above 15 °C (Austin & Austin, 1999; Larsen & Møllgaard, 1981). The bacterium is motile by means of a sheathed polar flagellum, and both the flagellum, motility and chemotaxis are important for virulence after infection of rainbow trout by immersion (McGee *et al.*, 1996; Milton *et al.*, 1996; Ormonde *et al.*, 2000; O'Toole *et al.*, 1996).

Flagellation and motility are of relative high cost to the bacterial metabolism and the stable maintenance of this system indicates that it is important for survival of the bacteria. As an inhabitant of the natural marine environment, *V. anguillarum* must survive changes in environmental factors such as salinity, temperature and availability of nutrients. Bordas *et al.* (1998) showed that *V. anguillarum* has a reduced chemotactic response towards fish mucus when exposed to 3.5% NaCl compared to 1.7% NaCl, although this was dependent on the source of the mucus. It is, however, not fully understood how environmental factors influence motility and/or chemotaxis

of *V. anguillarum*, or whether environmentally induced changes in motility influence the ability of the bacterium to cause disease.

The aim of this study was to determine the influence of temperature, salinity and starvation on the motility and chemotaxis of *V. anguillarum*. Serine was chosen as the attractant as it has previously been found in relatively high concentrations in mucus from rainbow trout and is a good attractant for *V. anguillarum* compared to other carbohydrates and amino acids present in mucus (O'Toole *et al.*, 1999). The role of pre-adaptation to different conditions on the chemotactic response was also investigated. The flagellar motor of other marine vibrios (e.g. *Vibrio cholerae*, *Vibrio parahaemolyticus* and *Vibrio alginolyticus*) is powered by an electrochemical gradient of Na⁺ across the cytoplasmic membrane (Atsumi *et al.*, 1992; Chernyak *et al.*, 1983; Kawagishi *et al.*, 1995; Kojima *et al.*, 1999a). If this were also the case in *V. anguillarum*, it would provide a direct mechanism whereby a change in Na⁺ concentration would influence chemotactic motility. We therefore investigated the dependence of *V. anguillarum* flagellum rotation on Na⁺ during growth and starvation.

The experiments show that *V. anguillarum* is motile and chemotactic even after starvation. Since motility and chemotaxis are important virulence factors, it was interesting to test whether the ability of the organism to cause disease was also unaffected by this treatment. We therefore tested whether starvation, which is a common situation for marine bacteria (Östling *et al.*, 1993), affects virulence of *V. anguillarum*.

METHODS

Bacterial strain and media. *V. anguillarum* NB10, a clinical isolate from Sweden (Norqvist *et al.*, 1989), was used throughout this study. Cultures of *V. anguillarum* were grown and maintained in Tryptic soy broth (TSB) (Difco) supplemented with 1% (w/v) NaCl (final concentration of NaCl, 1.5%) (TSB-1.5) or on Tryptic soy agar plates (Difco) supplemented with 1% NaCl (TSA-1.5). In some experiments, the bacteria were grown in TSB containing 0.5, 3.0 or 4.5% NaCl (TSB, TSB-3.0, TSB-4.5). The bacteria were maintained in the exponential growth phase for approximately eight generations before use. Glycerol (15%, v/v) stocks of the strain were kept at -80 °C for long-term storage.

Growth and stress conditions. To study the role of temperature on chemotaxis, the bacteria were cultured and the chemotaxis assay (see below) was performed at 5, 15 and 25 °C. All equipment and liquids were adjusted to the actual temperature before use. The role of salinity on motility and chemotaxis was investigated on cells grown in TSB-1.5 and TSB-3.0 at 25 °C. Bacteria used for measurements of motility and chemotaxis were harvested from exponentially growing cultures by centrifugation at 4000 r.p.m. for 5 min, washed twice in PBS [130 mM NaCl (~0.8%), 10 mM NaPO₄, pH 7.2] and resuspended in PBS to 1×10^8 c.f.u. ml⁻¹. Cells grown in TSB-3.0 were washed and resuspended in PBS with 2.0% NaCl. Thereafter the bacteria were diluted 1:9 in chemotaxis buffer (PBS, 0.01 mM EDTA) containing 0.8, 2.0, 3.0 and 4.0% (w/v) NaCl. For the starvation experiments, the bacteria were diluted in PBS (multiple nutrient limitations) and thereafter left at 25 °C for 2 and 8 days. The number of c.f.u. was determined in all bacterial suspensions by spreading serial dilutions on TSA-1.5 prior to and after 1 h incubation in chemotaxis buffer with different salinity and temperature. Total cell numbers were counted by using a Zeiss Axioplane epifluorescence microscope after polycarbonate membrane filtration and acridine orange staining (Binnerup *et al.*, 1993).

Chemotaxis assay. The technique used was the capillary assay (Adler, 1973) with a few modifications as described previously (Larsen *et al.*, 2001). Briefly, suspensions of washed cells were dispensed in 0.4 ml quantities to 1.5 ml Eppendorf tubes. Capillaries (disposable 5 µl pre-calibrated pipettes) (Vitrex) filled with 10 mM serine diluted in chemotaxis buffer were immersed into the bacterial suspension. Capillary tubes filled with the same buffer as used for dilution of the bacteria were included as controls. Data shown represent the mean of at least three separate experiments, each done in duplicate. The same number of c.f.u. ml⁻¹ was used in all assays, except for cells starved for 8 days and exposed to 2.7% NaCl. To normalize differences in motility and experimental variations, the chemotactic response is expressed as the ratio of the number of bacteria in attractant capillaries to that in control capillaries. A ratio of 2 or greater is considered significant (Hazen *et al.*, 1984). The data were analysed statistically by analysis of variance (one-way ANOVA). Bacterial counts were log transformed before the data were analysed in order to achieve a uniform variance.

Motility assay. Motility was measured by digitized image analysis. A standard video camera (JAI 2040) was mounted on a Zeiss

Axioplan microscope. To study the role of salinity and starvation, *V. anguillarum* was sucked up in a microslides capillary tube, viewing path length 0.4 mm (Camlab) and examined by dark-field microscopy (10×/0.30 plan neofluar objective). Ten seconds of each video sequence was subsequently digitized (25 images s⁻¹, PAL format). Mean run, and mean and maximum velocity were determined using the tracking system LABTRACK (Dimedia) (Thar *et al.*, 2000). Swimming tracks were recorded two-dimensionally but all tracks were detected. Thus, to ensure that the tracks used for calculations are almost parallel to the optical plane of the microscope (to avoid interference of bacteria swimming through the focal plane), only tracks above a certain length and velocity were used. The threshold levels for minimum length per track and minimum velocity were set to 0.36 s and 8 µm s⁻¹, respectively. Number of motile cells analysed per video sequence ranged from 148 to 282 cells with exponential phase cells and from 32 to 192 with starved cells. To study the role of temperature on swimming speed, *V. anguillarum* cells growing exponentially in TSB-1.5 at 5, 15 and 25 °C were examined by phase-contrast microscopy (100×/1.30 plan neofluar objective). A video was recorded and 15 s subsequently digitized (12.5 images s⁻¹, PAL format). The observation temperature was the same as the incubation temperature. The mean swimming speed was obtained by measuring at least 20 runs of cells by use of the AXIOVISION 3.0 image analysis program (Carl Zeiss).

Na⁺-dependence of the flagellar motor. Phenamil was used to study the dependence of rotation of the flagellum on Na⁺ as described by Atsumi *et al.* (1990) and Kojima *et al.* (1999a, b). Different concentrations (5–50 µM) of phenamil (Sigma) were added to growing bacteria after wash and resuspension in PBS and to bacteria starved in PBS at 25 °C for 2 and 8 days. Phenamil was added immediately before motility analysis. The swimming speed was measured as described above to study the role of temperature.

Fish infections. Rainbow trout (*Oncorhynchus mykiss* L.) with a body weight of 1.3–3.2 g (mean 2.4 g) were kept in 100 l capacity tanks containing aerated, re-circulating tap water supplemented with 0.6% NaCl at 12 °C. The fish were infected by immersion with either exponentially growing cells washed twice in PBS or bacteria starved for 2 days in PBS. The bacteria were added in 10-fold dilutions to 1 litre of diluted M9 minimal medium [g (1 tap water)⁻¹ after dilution: Na₂HPO₄, 3.94; KH₂PO₄, 0.923; NaCl, 0.31; NH₄Cl, 0.31]. Seven fish were exposed to each bacterial dilution. The fish were removed after 30 min, dipped briefly in tap water before they were transferred to a tank containing 30 l of aerated, re-circulating water. Fish were monitored daily. Dead fish were removed and subjected to bacteriological examination. Bacteria recovered from the kidney on Marine agar (Difco) supplemented with 5% bovine blood were subjected to a slide agglutination test to confirm identity. The antiserum used was a polyclonal antiserum raised in rabbits against *V. anguillarum* serotype O1 (Larsen *et al.*, 1994). The 50% lethal dose (LD₅₀) was calculated according to the statistical method of Reed & Muench (1938). The experiment was repeated once. The experiment was done in accordance with the legal notices of the Danish Animal Experiments Inspectorate.

RESULTS

Chemotactic response at different temperatures

Temperature had a marked effect on the chemotactic response of *V. anguillarum* to serine (Table 1). The highest chemotactic response was seen when the chemotaxis assay was performed at 25 °C. The ratio between the number of c.f.u. in the attractant capillary to the number in the control capillary at 5 and 15 °C was only about 2 and 21% of the

Table 1. Role of temperature on the chemotactic response of *V. anguillarum*

The chemotactic response was determined by the capillary tube method (see Methods). Bacteria were grown to exponential phase at 5, 15 or 25 °C; the chemotaxis assay was done at 5, 15, 25 or 37 °C.

Temperature (°C) during:		Chemotactic response		
Growth	Assay	Serine (c.f.u. per capillary)	Control (c.f.u. per capillary)	Ratio*
5	5	2 531 ± 597	663 ± 180	4 ^a
15	5	3 656 ± 882	641 ± 189	7 ^a
25	5	3 937 ± 840	1 405 ± 291	3 ^a
5	15	14 402 ± 2 154	633 ± 176	25 ^b
15	15	21 753 ± 10 876	298 ± 68	100 ^b
25	15	15 912 ± 3 206	543 ± 111	35 ^b
5	25	52 671 ± 15 002	208 ± 61	291 ^c
15	25	92 763 ± 23 439	396 ± 100	233 ^c
25	25	91 667 ± 13 135	252 ± 19	363 ^c
25	37	35 350 ± 1 668	878 ± 162	45 ^b

*Ratio between c.f.u. in the serine capillary to c.f.u. in the control capillary without attractant. Groups with different letters are significantly different from each other ($P < 0.05$).

ratio at 25 °C, respectively. The chemotactic ratio was significantly lower at 37 °C than at 25 °C. There was no significant difference between the number of c.f.u. in the bacterial suspension determined immediately after resuspension in chemotaxis buffer and after 1 h incubation (data not shown).

Chemotactic response at different salinities

The chemotactic response to serine was significantly decreased when the NaCl concentration in the chemotaxis buffer was raised from 0.8 to 1.8, 2.7 or 3.6 % (Table 2). For cells grown in TSB-1.5, the chemotactic response in 1.8 % NaCl was reduced to 1/20 of the highest response at 0.8 % NaCl, and at 2.7 % NaCl the chemotactic ratio was 1.5 % of the maximum ratio. The chemotactic response in 0.8 % NaCl was significantly lower when the bacteria were grown in 3 % NaCl prior to the assay, compared to growth in 1.5 % NaCl. When the concentration of NaCl in the chemotaxis buffer was 2.7 and 3.6 %, the chemotactic response was very low or there was no response (ratio below 2) irrespective of the concentration of NaCl in the growth medium (Table 2). Incubation of *V. anguillarum* in chemotaxis buffer with the different salinities did not influence the culturability within the monitored time period (1 h) (data not shown).

Chemotactic response after pre-incubation at low temperature and high salinity

To investigate if the reduced chemotactic response at low temperatures and at high salinity was caused partly by transient changes triggered by temperature or osmotic shift, the chemotactic response of *V. anguillarum* was determined after pre-incubation for 2.5 h. *V. anguillarum* was grown in TSB-1.5 at 25 °C and incubated either in chemotaxis buffer at 5 °C or in chemotaxis buffer with 2.7 % NaCl for 2.5 h before the chemotaxis assay. The responses after pre-incubation were not different from the responses of washed exponentially growing cells (data not shown). This indicates that the reduced responses were not caused by transient changes in the cells.

Table 2. Role of NaCl concentration on the chemotactic response of *V. anguillarum* at 25 °C

The chemotactic response was determined by the capillary tube method (see Methods). Bacteria were grown to exponential phase in TSB-1.5 or TSB-3.0, washed and resuspended in chemotaxis buffer with 0.8, 1.8, 2.7 or 3.6 % NaCl. The number of c.f.u. ml⁻¹ was standardized to 1×10^7 .

[NaCl] (%) in growth medium	[NaCl] (%) in chemotaxis buffer	Chemotactic response		
		Serine (c.f.u. per capillary)	Control (c.f.u. per capillary)	Ratio*
1.5	0.8	83 860 ± 10 668	254 ± 21	334 ^a
	1.8	6 450 ± 479	479 ± 94	14 ^b
	2.7	763 ± 350	155 ± 35	4 ^c
	3.6	290 ± 60	BD	—
3.0	0.8	64 083 ± 23 487	465 ± 223	159 ^d
	2.7	4 498 ± 2 457	1 018 ± 367	4 ^c
	3.6	278 ± 64	177 ± 32	1.6 ^c

BD, Below the level of detection (detection limit was 100 c.f.u. per capillary).

*Ratio between c.f.u. in the serine capillary to c.f.u. in the control capillary. Groups with different letters are significantly different from each other ($P < 0.05$).

Table 3. Role of starvation on the chemotactic response of *V. anguillarum* at 25 °C

The chemotactic response was determined by the capillary tube method (see Methods). Bacteria were grown to exponential phase in TSB-1.5, washed and resuspended in chemotaxis buffer with 0.8 or 2.7% NaCl, or harvested in exponential growth phase in TSB-1.5 and resuspended in PBS and left for 2 and 8 days. The number of c.f.u. ml⁻¹ was standardized to 1 × 10⁷. The data from exponential phase are also presented in Table 2.

Growth phase	[NaCl] (%) in chemotaxis buffer	Chemotactic response		
		Serine (c.f.u. per capillary)	Control (c.f.u. per capillary)	Ratio*
Exponential	0.8	83 860 ± 10 668	254 ± 21	334 ^a
Exponential	2.7	763 ± 350	155 ± 35	4 ^b
Starved for 2 days	0.8	170 000 ± 8 660	823 ± 168	207 ^c
Starved for 2 days	2.7	601 ± 164	546 ± 170	1 ^d
Starved for 8 days	0.8	93 433 ± 29 064	463 ± 140	196 ^c
Starved for 8 days†	0.8	6 600 ± 283	105 ± 4	63†
Starved for 8 days†	2.7	BD	BD	ND

ND, Not determined; BD, below the level of detection (detection limit was 100 c.f.u. per capillary).

*Ratio between the number of c.f.u. in the serine capillary to c.f.u. in the control capillary. Groups with different letters are significantly different from each other ($P < 0.05$).

†The number of c.f.u. ml⁻¹ in these experiments was 7 × 10⁵. These results were therefore omitted from the statistical analysis.

Chemotactic responses during starvation

The capillary assay demands that approximately the same number of culturable cells is used in each assay. To ensure that this was achievable in studies using starved cells, the total number of cells (TC) and the number of c.f.u. was initially followed during starvation. There was no difference between TC and the number of c.f.u. at the onset of starvation and after 2 days starvation. During this period, numbers increased approximately 17% to 2.2 × 10⁸ most likely because *V. anguillarum* is known to perform reductive cell divisions in the transition from growth to starvation, i.e. the number of bacteria increases without a corresponding increase in biomass (Kjelleberg *et al.*, 1987; Nelson *et al.*, 1997). From day 2 to day 8, TC was reduced by 27% to 1.5 × 10⁸ and the number of c.f.u. was reduced by 91% to 1.7 × 10⁷. Thus, the fraction of culturable cells (TC/c.f.u.) was 11% after 8 days starvation. In general, the number of c.f.u. after 8 days varied between 3 and 11% of the maximum number of culturable cells (day 2).

The starved bacteria showed a high chemotactic response, but the response was significantly lower than the response of non-starved cells (one-way ANOVA, $P = 0.04$) (Table 3). Although the chemotactic response (measured as ratio) was decreased, the number of c.f.u. in the capillary (serine and control) was higher after 2 days starvation. The reason for this is unknown but changed surface properties of the bacteria may be involved. Bacteria starved for 2 days in PBS were not able to make a significant chemotactic response at 2.7% NaCl (ratio < 2) and after 8 days starvation the chemotactic response at 2.7% NaCl was below the level

of detection (Table 3). The culturability of *V. anguillarum* was not influenced by incubation for 1 h in chemotaxis buffer with 2.7% NaCl (data not shown).

Effects of temperature on motility

Cells of *V. anguillarum* were motile at all growth temperatures tested, and also after temperature up- and down-shift as observed by microscopy. The swimming speed increased significantly from 25 μm s⁻¹ at 5 °C to 36 and 40 μm s⁻¹ at 15 and 25 °C, respectively (one-way ANOVA, $P = 0.002$).

Effects of NaCl on motility

The maximum velocity of the motile cells was higher when measured in the growth medium than after washes and resuspension in PBS (Table 4). However, only a few bacteria in each sample reached the maximum velocity (data not shown). The velocity seemed unaffected by the concentration of NaCl in the medium. The relatively high standard deviation between cells in one sample showed that the velocity of cells within the same population is very heterogeneous (Table 4). The mean time of runs was 0.51 s in all cases.

The possible role of Na⁺ in the generation of power for motility was studied by adding phenamil to cultures of *V. anguillarum* in exponential growth phase and after starvation for 2 and 8 days. Phenamil reduced the swimming speed, and at a concentration above 20 μM the bacteria became non-motile (Table 5). The growth rate of

Table 4. Effect of NaCl on motility of *V. anguillarum*

Motility was determined at 25 °C as described in Methods during growth in TSB-1·5 or TSB-3·0, or after harvesting and resuspension in PBS with different concentration of NaCl (0·8, 1·8, 2·7 or 3·6). Data from one experiment are shown. The SD between samples was below 2.

Growth medium	Resuspension medium	Mean velocity* ($\mu\text{m s}^{-1}$)	Max. velocity ($\mu\text{m s}^{-1}$)
TSB-1·5	–	20 ± 9	48
TSB-3·0	–	20 ± 8	40
TSB-1·5	PBS-0·8	18 ± 6	36
TSB-1·5	PBS-1·8	16 ± 6	41
TSB-1·5	PBS-2·7	15 ± 4	28
TSB-1·5	PBS-3·6	13 ± 3	26
TSB-3·0	PBS-0·8	16 ± 5	34
TSB-3·0	PBS-2·7	20 ± 7	37
TSB-3·0	PBS-3·6	15 ± 5	38

*Values represent mean velocity of the motile cells ± SD within one sample.

V. anguillarum in TSB-1·5 at 25 °C was not affected by the addition of 5, 10, 20 or 50 μM phenamil (data not shown).

Motility after starvation

Cells of *V. anguillarum* were still motile after 2 and 8 days starvation as seen from the number of c.f.u. in the control capillaries (Table 3) and from microscopic examination. The data from the motility analysis showed that the velocity of the motile cells did not vary between the different treatments (Table 6). However, the standard deviation between cells in one sample was relatively high. The mean length of runs was 0·51 s in all samples. The mean number of motile cells was about 80 % for exponentially growing

Table 5. Effect of phenamil on motility of *V. anguillarum*

Phenamil was added to cells of *V. anguillarum* in exponential growth phase or starved for 2 and 8 days in PBS. The mean swimming speeds were obtained for at least 20 cells immediately after addition of phenamil. Exp., exponential.

Physiological state	[Phenamil] (μM)	Swimming speed ($\mu\text{m s}^{-1}$)
Exp. growth phase	0	40
Starved for 2 days	0	37
Starved for 8 days	0	29
Exp. growth phase	5	12
Exp. growth phase	20	3
Exp. growth phase	50	0
Starved for 2 days	20	0
Starved for 8 days	20	0

cells of *V. anguillarum* NB10, but after washing and resuspension in chemotaxis buffer the motile fraction was reduced to about 40 %. This probably results from the fragile character of the flagellar filament, resulting in shearing of parts of the flagellum (Kojima *et al.*, 1999a). After starvation for 8 days the fraction of motile cells was 6 %. Transferring cells starved for 8 days to 2·7 % NaCl reduced the motile fraction to less than 1 %, whereas the fraction was unaffected when transferred to 0·8 % NaCl.

Pathogenicity of starved cells

As the starved cells showed a very high chemotactic response, and as chemotactic motility is a virulence factor (O'Toole *et al.*, 1996), it was interesting to test if the virulence of starved cells was comparable to that of growing cells. Comparison of pathogenicity between growing and starved cells of *V. anguillarum* showed that starved cells were at least as virulent as growing cells. The LD₅₀ of exponentially grown and 2 days starved bacteria was $6\cdot9 \times 10^6$ and $2\cdot5 \times 10^6$ c.f.u. ml⁻¹, respectively. The challenge isolate of *V. anguillarum* serogroup O1 was isolated in pure culture from the kidney of all dead fish. No fish from the control group died during the experiment and it was not possible to isolate *V. anguillarum* from fish maintained in the control aquarium.

DISCUSSION

The results of this study showed that the optimal temperature for chemotaxis of *V. anguillarum* to serine was 25 °C, which corresponds to the optimal growth temperature for the bacterium. Outbreaks of vibriosis occur when water temperatures exceed 15 °C (Austin & Austin, 1999; Larsen & Mellergaard, 1981). However, *V. anguillarum* was chemotactic at temperatures as low as 5 °C indicating that the absence of vibriosis in the cold season is not due to the

Table 6. Motility of *V. anguillarum* after starvation and of starved cells exposed to high concentrations of NaCl

Bacteria were grown in TSB-1·5, washed and resuspended in PBS and left for 2 and 8 days. Before the chemotaxis assay the bacterial suspensions were diluted 1:9 in PBS or PBS with 3 % NaCl (+3 % NaCl). Motility was analysed as described in Methods.

Treatment	Mean velocity* ($\mu\text{m s}^{-1}$)	Max. velocity ($\mu\text{m s}^{-1}$)
Onset of starvation	18 ± 6	36
Onset of starvation + 3 % NaCl	15 ± 4	28
Starvation, 2 days	16 ± 6	34
Starvation, 8 days	14 ± 4	26
Starvation, 2 days + 3 % NaCl	14 ± 4	28
Starvation, 8 days + 3 % NaCl	13 ± 3	29

*Values are mean swimming speed of the motile cells ± SD in one sample. The SD between samples was below 2.

absence of the flagellum, motility and chemotactic ability. We tested only the role of temperature and salinity on growing cells; starved bacteria may react differently to low temperatures or high salinity.

The swimming speed of *V. anguillarum* was reduced at lower temperatures. A similar temperature dependency has been described for *Salmonella enterica* serovar Typhimurium (Maeda *et al.*, 1976; Miller & Koshland, 1977). The relationship between viscosity and temperature may account for this. The viscosity of pure water at 5 and 25 °C is 1·519 and 0·890 centipoises, respectively, a change by a factor of 1·6 (Weast & Astle, 1983). This corresponds to the difference in swimming velocity found at the two temperatures in the present study, and most likely flagellar power-generation is the same at the different temperatures.

The decrease in chemotactic response at decreasing temperatures was not only due to reduced swimming speed as shown by the change in chemotactic ratio between attractant and control capillaries. This allowed us to conclude that temperature has a more pronounced effect on chemotaxis than on motility. This is in accordance with reports on *Escherichia coli*, where a raise in temperature from 20 to 30 °C results in a 20-fold increase in chemotactic response, but only a two- to threefold increase in motility (Adler, 1973). The reason for the temperature dependency of chemotaxis is not known. However, the rate of phosphorylation and/or (de-)methylation of signal transduction proteins and receptors may increase with higher temperature, like the kinetics of other enzymic reactions. Furthermore, the fluidity of membranes depends on temperature. This may also include the sheath of the flagellum of *V. anguillarum*, as the flagellar sheath is a distinct outer-membrane domain (McCarter, 2001).

The chemotactic responses to serine were also influenced by salinity, and primarily by the salinity of the chemotaxis buffer compared to the salinity of the growth medium. Thus, the chemotactic responses were significantly reduced when the salinity of the chemotaxis buffer increased. Likewise, data from another study indicate that the highest chemotactic response of *V. anguillarum* to fish mucus was never obtained at a salinity of 3·5 ‰ compared to 1·0 and 1·7 ‰ (Bordas *et al.*, 1998). *V. anguillarum* is a halophilic bacterium causing infections in the marine environment and survives starvation in sea water microcosm with 1·0–3·5 ‰ NaCl for up to 4 weeks (Hoff, 1989). Padan & Krulwich (2000) speculated that Na⁺ interferes with optimal protein folding, and in some bacteria Na⁺-dependent gene expression has been shown, for example in *V. cholerae* (Hase & Mekalanos, 1999). A plausible explanation for the dependency on salinity of chemotactic responses of *V. anguillarum* is that proteins of the signal transduction pathway are affected in a Na⁺-dependent manner. However, more detailed studies are needed to elucidate the role of Na⁺ in chemotaxis.

Pre-adaptation to low temperature or high osmolarity did

not alter the influence of these parameters on chemotaxis. This was investigated by pre-incubation for 2·5 h. This incubation time was chosen based on information from other bacteria, as the synthesis of specific stress proteins of *V. anguillarum* has not been investigated. Most of the stress and starvation proteins in *Salmonella*, *Pseudomonas putida* and *Vibrio* sp. strain S14 are synthesized during the first 2 h following exposure to stress conditions (Nyström *et al.*, 1990; Spector, 1998; Givskov *et al.*, 1994), and many of the structural and physiological changes which are triggered by osmotic shifts in *E. coli*, such as cell dehydration, decreased cytoplasmic water activity and reduced cell respiration, are restored within the first hour after a shift (Wood, 1999). However, the starvation-stress response of *V. anguillarum* has been shown to differ from these bacteria (Nelson *et al.*, 1997) and it may be that the time chosen does not correspond to the time that *V. anguillarum* requires to induce the stress response.

By video analysis it was found that individual cells of *V. anguillarum* had similar motility characteristics with respect to velocity, acceleration and duration of runs in PBS containing 0·8–3·6 ‰ NaCl. This is in accordance with a study of *V. alginolyticus* showing that the swimming speed is independent of the NaCl concentration when it is above 100 mM (0·6 ‰) (Yoshida *et al.*, 1990). The results indicate that the rotation rate of the flagellum is independent of a concentration of NaCl between 0·8 and 3·6 ‰, the concentrations used in this study.

The swimming speed of bacteria that use Na⁺ to drive their flagellar motors is reduced by the addition of phenamil, which binds specifically to the Na⁺ channel of the flagellar motor and inhibits its rotation (Atsumi *et al.*, 1990; Kojima *et al.*, 1999a). The swimming speed of non-starved *V. anguillarum* cells was decreased in the presence of phenamil, indicating that Na⁺ drives the flagellum of *V. anguillarum* as in other marine vibrios, for example *V. cholerae*, *V. parahaemolyticus* (Atsumi *et al.*, 1992) and *V. alginolyticus* (Chernyak *et al.*, 1983; Kawagishi *et al.*, 1995; Kojima *et al.*, 1999a). Here we also observed that the swimming speed of starved *V. anguillarum* cells was decreased by phenamil, indicating that *V. anguillarum* is able to maintain a Na⁺ gradient sufficient to rotate the flagellum at maximum speed during 8 days starvation. Maintenance of a sizeable membrane potential, at least during short-term starvation (2 days), has previously been shown in *Vibrio fluvialis* (Smigielski *et al.*, 1989).

During starvation, the culturable and motile fractions of *V. anguillarum* were reduced, and after 8 days the motile fraction roughly corresponded to the culturable fraction. Furthermore, the starved cells displayed a strong chemotactic response to serine. However, in marine *Vibrio* sp. strain S14 no chemotactic response could be detected after 24 h starvation, and after 3 days the motile fraction was only 5% although the culturable fraction was approximately 100% (Malmcrona-Friberg *et al.*, 1990). This loss of motility during starvation corresponded to a loss of

flagella (Malmcrona-Friberg *et al.*, 1990). Thus, it seems that these two marine vibrios have different starvation survival strategies.

We showed that transferring cells of *V. anguillarum* prior to the chemotaxis assay from 0.8 to 2.7% NaCl in chemotaxis buffer gave a larger reduction in chemotactic ability after 2 days starvation than at the onset of starvation. In other words, the chemotactic response of starved cells of *V. anguillarum* was more sensitive to high concentrations of NaCl than growing cells. This suggests that *V. anguillarum* does not develop cross-resistance to osmotic stress during starvation or that the resistance is transient. This is in accordance with a previous report that has shown that *V. anguillarum* does not develop cross-resistance against heat shock during starvation and that the cross-resistance to oxidative stress disappears after 5 days starvation (Nelson *et al.*, 1997). Also, motility of cells starved for 8 days was affected by 2.7% NaCl. There seems to be a complex relationship between Na⁺ and other stresses such as temperature and pH (Padan & Krulwich, 2000).

Cells of *V. anguillarum* that maintain motility during starvation had the same motility characteristic with respect to velocity, acceleration and duration of runs as growing cells. This indicates that at least a fraction of starved cells of *V. anguillarum* maintain a motive force that is sufficient to drive the rotation of the flagellum at levels corresponding to the rate of rotation during growth. This is different from *E. coli*, where a decrease in swimming speed was found when the cells entered the stationary phase, most likely due to a decreased proton motive force (Amsler *et al.*, 1993).

A hallmark of bacteria is their ability to survive and respond to changes in their surroundings. Motility and chemotaxis are also influenced by such changes. This study showed that *V. anguillarum* is motile and chemotactic at many different conditions of relevance to its natural environment. Temperature and salinity, however, affect the phenotype of *V. anguillarum*, and the growth conditions prior to the assay were of less importance than the incubation conditions at the time of the measurements of the chemotactic ability. *V. anguillarum* was able to elicit a high chemotactic response during starvation. In nutrient-deprived environments, it may be important for the survival of bacteria that they are able to localize themselves in microenvironments with the best conditions for growth (Blackburn *et al.*, 1998). Furthermore, we showed that *V. anguillarum* cells starved for 2 days were as virulent as exponentially growing cells after bath challenge. This is similar to what has been shown in *Aeromonas hydrophila* to a degree, where cells starved for 1 day were more virulent than cells in exponential growth phase after intraperitoneal injections (Rahman *et al.*, 1997). Also, starved cells of *V. vulnificus* and *Pasteurella piscicida* have been shown to retain a degree of virulence similar to that of non-starved cells after intraperitoneal inoculations into

eels and turbot, respectively (Biosca *et al.*, 1996; Magarinos *et al.*, 1994). The role of starvation for regulation of virulence genes in *V. anguillarum* has not been studied yet. However, the stationary-phase sigma factor RpoS is known to control a large group of genes including genes important for virulence in other species, for example *V. cholerae* and *S. enterica* serotype Typhimurium (Spector, 1998; Yildiz & Schoolnik, 1998). Thus, it seems that under certain conditions non-growth prior to contact with the host does not interfere with the ability of the bacterium *V. anguillarum* to cause infection.

ACKNOWLEDGEMENTS

The Danish Biotek II programme (grant no. 9502025) supported this work. We thank Dr Roland Thar, Marine Biological Laboratory, University of Copenhagen, Denmark for his help with the motility analysis and Dr Svend Binnerup for helpful criticism of the manuscript. Dr Debra Milton, University of Umeå, Sweden kindly provided *V. anguillarum* strain NB10 and the antiserum against the flagellins.

REFERENCES

- Adler, J. (1973).** A method for measuring chemotaxis and use of the method to determine optimum conditions for chemotaxis by *Escherichia coli*. *J Gen Microbiol* **74**, 77–79.
- Amsler, C. D., Cho, M. & Matsumura, P. (1993).** Multiple factors underlying the maximum motility of *Escherichia coli* as cultures enter post-exponential growth. *J Bacteriol* **175**, 6238–6244.
- Atsumi, T., Sugiyama, S., Cragoe, E. J., Jr & Imae, Y. (1990).** Specific inhibition of the Na⁺-driven flagellar motors of alkalophilic *Bacillus* strains by the amiloride analog phenamil. *J Bacteriol* **172**, 1634–1639.
- Atsumi, T., McCarter, L. & Imae, Y. (1992).** Polar and lateral flagellar motors of marine *Vibrio* are driven by different ion-motive forces. *Nature* **355**, 182–184.
- Austin, B. & Austin, D. A. (1999).** *Bacterial Fish Pathogens: Disease of Farmed and Wild Fish*, 3rd edn, pp. 238–240. Chichester: Praxis.
- Binnerup, S. J., Jensen, D. F., Thordal-Christensen, H. & Sørensen, J. (1993).** Detection of viable, but non-culturable *Pseudomonas fluorescens* DF57 in soil using a microcolony epifluorescence technique. *FEMS Microbiol Ecol* **12**, 97–105.
- Biosca, E. G., Amaro, C., Marco-Noales, E. & Oliver, J. D. (1996).** Effect of low temperature on starvation-survival of the eel pathogen *Vibrio vulnificus* biotype 2. *Appl Environ Microbiol* **62**, 450–455.
- Blackburn, N., Fenchel, T. & Mitchell, J. (1998).** Microscale nutrient patches in planktonic habitats shown by chemotactic bacteria. *Science* **282**, 2254–2256.
- Bordas, M. A., Balebona, M. C., Rodriguez-Maroto, J. M., Borrego, J. J. & Morinigo, M. A. (1998).** Chemotaxis of pathogenic *Vibrio* strains towards mucus surfaces of gilt-head sea bream (*Sparus aurata* L.). *Appl Environ Microbiol* **64**, 1573–1575.
- Chernyak, B. V., Dibrov, P. A., Glagolev, A. N., Sherman, M. Y. & Skulachev, V. P. (1983).** A novel type of energetics in a marine alkali-tolerant bacterium $\Delta\mu_{\text{Na}}$ -driven motility and sodium cycle. *FEMS Microbiol Lett* **164**, 38–42.
- Givskov, M., Eberl, L., Møller, S., Poulsen, L. K. & Molin, S. (1994).** Responses to nutrient starvation in *Pseudomonas putida* KT2442: analysis of general cross-protection, cell-shape, and macromolecular content. *J Bacteriol* **176**, 4816–4824.

- Hase, C. C. & Mekalanos, J. J. (1999). Effects of changes in membrane sodium flux on virulence gene expression in *Vibrio cholerae*. *Proc Natl Acad Sci U S A* **96**, 3183–3187.
- Hazen, T. C., Dimock, R. V., Esch, G. W., Mansfield, A. & Raker, M. L. (1984). Chemotactic behaviour of *Aeromonas hydrophila*. *Curr Microbiol* **10**, 13–18.
- Hoff, K. A. (1989). Survival of *Vibrio anguillarum* and *Vibrio salmonicida* at different salinities. *Appl Environ Microbiol* **55**, 1775–1786.
- Kawagishi, I., Maekawa, Y., Atsumi, T., Homma, M. & Imae, Y. (1995). Isolation of the polar and lateral flagellum-defective mutants in *V. alginolyticus* and identification of their flagellar driving energy sources. *J Bacteriol* **177**, 5158–5160.
- Kjelleberg, S., Hermansson, M., Mården, P. & Jones, G. W. (1987). The transient phase between growth and nongrowth of heterotrophic bacteria, with emphasis on the marine environment. *Annu Rev Microbiol* **41**, 25–49.
- Kojima, S., Yamamoto, K., Kawagishi, I. & Homma, M. (1999a). The polar flagellar motor of *Vibrio cholerae* is driven by an Na⁺ motive force. *J Bacteriol* **181**, 1927–1930.
- Kojima, S., Asai, Y., Atsumi, T., Kawagishi, I. & Homma, M. (1999b). Na⁺-driven flagellar motor resistant to phenamil, an amiloride analog, caused by mutations in putative channel components. *J Mol Biol* **285**, 1537–1547.
- Larsen, J. L. & Møllergaard, S. (1981). Microbiological and hygienic problems in marine aquaculture: furunculosis and vibriosis in rainbow trout (*Salmo gairdneri*, L.). *Bull Eur Assoc Fish Pathol* **1**, 29–31.
- Larsen, J. L., Pedersen, K. & Dalsgaard, I. (1994). *Vibrio anguillarum* serovars associated with vibriosis in fish. *J Fish Dis* **17**, 259–267.
- Larsen, M. H., Larsen, J. L. & Olsen, J. E. (2001). Chemotaxis of *Vibrio anguillarum* to fish mucus: role of the origin of the fish mucus, the fish species and the serogroup of the pathogen. *FEMS Microbiol Ecol* **38**, 77–80.
- Maeda, K., Imae, Y., Shioi, J. I. & Oosawa, F. (1976). Effect of temperature on motility and chemotaxis of *Escherichia coli*. *J Bacteriol* **127**, 1039–1046.
- Magarinos, B., Romalde, J. L., Barja, J. L. & Toranzo, A. E. (1994). Evidence of a dormant but infective state of the fish pathogen *Pasteurella piscicida* in seawater and sediment. *Appl Environ Microbiol* **60**, 180–186.
- Malmcrona-Friberg, K., Goodman, A. & Kjelleberg, S. (1990). Chemotactic responses of marine *Vibrio* sp. strain S14 (CCUG 15956) to low-molecular-weight substances under starvation and recovery conditions. *Appl Environ Microbiol* **56**, 3699–3704.
- McCarter, L. (2001). Polar flagellar motility of the *Vibrionaceae*. *Microbiol Mol Biol Rev* **65**, 445–462.
- McGee, K., Hörstedt, P. & Milton, D. L. (1996). Identification and characterization of additional flagellin genes from *Vibrio anguillarum*. *J Bacteriol* **178**, 5188–5198.
- Miller, J. B. & Koshland, D. E. (1977). Membrane fluidity and chemotaxis: effects of temperature and membrane lipid composition on the swimming behavior of *Salmonella typhimurium* and *Escherichia coli*. *J Mol Biol* **111**, 183–201.
- Milton, D. L., O'Toole, R., Hörstedt, P. & Wolf-Watz, H. (1996). Flagellin A is essential for the virulence of *Vibrio anguillarum*. *J Bacteriol* **178**, 1310–1319.
- Nelson, D. R., Sadlowski, Y., Eguchi, M. & Kjelleberg, S. (1997). The starvation-stress response of *Vibrio (Listonella) anguillarum*. *Microbiology* **143**, 2305–2312.
- Norqvist, A., Hagstrom, A. & Wolf-Watz, H. (1989). Protection of rainbow trout against vibriosis and furunculosis by the use of attenuated strains of *Vibrio anguillarum*. *Appl Environ Microbiol* **55**, 1400–1405.
- Nyström, T., Flårdh, K. & Kjelleberg, S. (1990). Responses to multiple-nutrient starvation in marine *Vibrio* sp. strain CCUG 15956. *J Bacteriol* **172**, 7085–7097.
- Ormonde, P., Hörstedt, P., O'Toole, R. & Milton, D. L. (2000). Role of motility in adherence to and invasion of a fish cell line by *Vibrio anguillarum*. *J Bacteriol* **182**, 2326–2328.
- Östling, J., Holmquist, L., Flårdh, K., Svenblad, B., Jouper-Jaan, Å. & Kjelleberg, S. (1993). Starvation and recovery of *Vibrio*. In *Starvation in Bacteria*. Edited by S. Kjelleberg. New York: Plenum.
- O'Toole, R., Milton, D. L. & Wolf-Watz, H. (1996). Chemotactic motility is required for invasion of the host by the fish pathogen *Vibrio anguillarum*. *Mol Microbiol* **19**, 625–637.
- O'Toole, R., Lundberg, S., Fredriksson, S. A., Jansson, A., Nilsson, B. & Wolf-Watz, H. (1999). The chemotactic response of *Vibrio anguillarum* to fish intestinal mucus is mediated by a combination of multiple mucus components. *J Bacteriol* **181**, 4308–4317.
- Padan, E. & Krulwich, T. A. (2000). Sodium stress. In *Bacterial Stress Responses*, pp. 117–130. Edited by G. Storz & R. Hengge-Aronis. Washington, DC: American Society for Microbiology.
- Rahman, M. H., Kawai, K. & Kusuda, R. (1997). Virulence of starved *Aeromonas hydrophila* to cyprinid fish. *Fish Pathol* **32**, 163–168.
- Reed, L. J. & Muench, H. (1938). A simple method of estimating fifty percent endpoints. *Am J Hyg* **27**, 493–497.
- Smigielski, A. J., Wallace, B. J. & Marshall, K. C. (1989). Changes in membrane functions during short-term starvation of *Vibrio fluvialis* strain NCTC 11328. *Arch Microbiol* **151**, 336–347.
- Spector, M. P. (1998). The starvation-stress response (SSR) of *Salmonella*. *Adv Microb Physiol* **40**, 233–279.
- Thar, R., Blackburn, N. & Kühn, M. (2000). A new system for three-dimensional tracking of motile microorganisms. *Appl Environ Microbiol* **66**, 2238–2242.
- Weast, R. C. & Astle, M. J. (1983). Table F40. In *Handbook of Chemistry and Physics*, 63rd edn. Boca Raton, FL: CRC Press.
- Wood, J. M. (1999). Osmosensing by bacteria: signals and membrane-based sensors. *Microbiol Mol Biol Rev* **63**, 230–262.
- Yildiz, F. H. & Schoolnik, G. K. (1998). Role of *rpoS* in stress survival and virulence of *Vibrio cholerae*. *J Bacteriol* **180**, 773–784.
- Yoshida, S., Sugiyama, S., Hojo, Y., Tokuda, H. & Imae, Y. (1990). Intracellular Na⁺ kinetically interferes with the rotation of the Na⁺-driven flagellar motors of *Vibrio alginolyticus*. *J Biol Chem* **265**, 20346–20350.