Influence of temperature, salinity, and pH on logistic growth rate of *Vibrio vulnificus* biotypes as determined by culture-based and real-time PCR methods.

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Introduction

Vibrio vulnificus is an opportunistic human pathogen responsible for septicemia and severe wound infections. *V. vulnificus* strains have been categorized into three biotypes determined by biochemical testing methods (Biosca et al.1997; Bisharat et al. 2005). Biotype 1 strains are most frequently responsible for human infections, while biotype 2 strains are associated with disease in eels (Amaro et al. 1999). Biotype 3 was identified in a series of outbreaks of human infections in Israel that were associated with handling of tilapia (Bisharat et al. 2005; Nudelman et al. 1997).

V. vulnificus is generally found in estuaries of temperate and tropical coastal waters. In its natural habitat, the bacterium faces incessantly fluctuating levels of nutrients, salinity and other aspects of its environment (Parvathi et al. 2004). Numerous studies observed the effects of selected physical variables on the survival of *V. vulnificus*; however, less is known about the optimal conditions for its growth. Furthermore, it is unclear whether interdependence among some variables, such as pH, temperature, and salinity exists. Environmental sampling often includes the documentation of local pH, but a relatively narrow range of values is generally found. The same is true for temperature and salinity. It has been documented that the species experiences reduced survival in the environment outside a certain temperature range (13-22°C) and in salinities above 25‰ (Kaspar et al. 1993). A microcosm study, however, suggested that optimal temperature for survival depends on salinity (Marco-Noales et al. 1999).

This study evaluated the influence of temperature, pH, and salinity on the growth rate of *V. vulnificus* biotypes 1, 2, and 3. Growth rate was assessed by two culture-dependent methods: measuring optical density at 600nm (OD), and plate counts, and total cell quantification by quantitative PCR (qPCR), targeting the *vvhA* gene (Campbell and Wright, 2003). Growth rates and generation times were calculated using the logistic equation. Growth rates were then compared and statistically analyzed among and between all physical variables and biotypes.

Materials and Methods

Strains and Media

One representative of each biotype was selected: CMCP6 (biotype 1), ATCC 33147 (biotype 2), and 302-99 (biotype 3). To accurately assess the salinity, including ionic strength, all cultures were grown in 50ml microcosms containing broth of defined minimal media (23.2mM Na_2HPO_4, 11.02mM KH_2PO_4, 9.34mM NH_4Cl, 1.0mM $MgSO_4$) augmented with 0.06% casamino acids and 0.006% yeast extract. Glucose was provided as a carbon source at a final concentration of 4.5mM. Salinity was adjusted by NaCl. Overnight cultures were grown in CAYEG broth (Tamplin and Colwell, 1986).

Physical variables and growth conditions

Cells from overnight cultures were washed with 1X PBS (pH 7) and resuspended in the minimal broth medium at approximately 10^7 CFU/ml. Microcosm cultures of each biotype, agitated at 155 rpm, were grown in triplicate and the experiment was repeated to provide six replicates at salinities of 5, 10, 15, 20, 25, 30, 35, and 40ppt. Growth at each salinity was

observed at 37, 30, and 25°C, and pH 7and pH 8 for total of 48 treatment/level combinations. The pH level was adjusted at each OD reading (approx. 20 min. intervals) with 300mM NaOH.

Growth observation

At each time point the optical density (OD) was measured at 600 nm for all samples using a NanoDrop 2000c (Thermoscientific, Inc). Plating was performed on TSA supplemented with 0.5%NaCl (TSA-NaCl) media during the exponential phase of growth to assess the relationship between OD and plate counts.

For each temperature and pH combination, the salinity with the optimal growth rate was chosen for additional analysis by qPCR. A one milliliter aliquot was boiled at 100°C for 10 minutes to lyse the cells. The product of boiling lysis served as template DNA for qPCR with an ABI 7500 Real Time PCR System (Applied Biosystems, Inc.). SYBR green I reagents (Applied Biosystems, Inc.) were used to visualize the increase in amplicon concentration using previously published primers and procedure (Campbell and Wright 2003). For these cultures several time points during the exponential growth phase were chosen for enumeration by plate counts to confirm growth consistent with other trials.

Analyses

Relationships between OD, concentrations determined by plate count and total cell concentrations determined by qPCR were calculated on $\log_{10}$-transformed data sets. For all trials, these data were plotted and self-start logistic curves were fitted using the nonlinear regression function of R 2.10.1 software (R Development Core Team, 2008; Liaw and Wiener, 2002; available http://cran.r-project.org/). Maximum logistic growth rates and generation times were determined and compared (Perni S., 2005). Three-way ANOVA was used to identify statistically significant effects of all three variables and their interactions (Minitab®Statistical software). The data were further analyzed by one-way ANOVA with post-hoc testing (Tukey-Kramer) to better identify statistical significance of individual salinities within each temperature/pH group (Graphpad Prism version 5).

Results and Discussion

The relationships between optical density, plate count measurements and qPCR measurements were established. Visual comparison between the data and their respective calculated logistic curves of all three *V. vulnificus* biotypes saw reasonably good fits with the data. The individual biotypes grew to different levels of culturable (CFU/ml) and total cell concentrations, and average total cell concentrations were always higher than corresponding plate counts. Overall, the total cell concentrations determined by qPCR were 2 to 12times higher than those of plate counts. As growth progressed to exponential and stationary phase, the magnitude of these discrepancies decreased. This result was expected due to the anticipated initial presence of dead cells, free DNA and viable but not culturable (VBNC) cells which would contribute to the total cell concentration detectable by qPCR but not plate counts. Furthermore, growth rates and generation times calculated using the relationship between plate counts and optical density were significantly higher than those determined using the relationship between qPCR and OD ($p<0.05$) for all biotypes. Interestingly, biotype 1 grew significantly faster than biotypes 2 and 3 and biotype 2 grew faster than biotype 3(all $p<0.001$) when plate counts or total cells were used for growth rate calculations.

The genome of *V. vulnificus* consists of two chromosomes. It has been previously suggested that the presence of two chromosomes could contribute to the bacterium's rapid reproduction rate (Yamaichi et al. 1999). During our study, the lowest average generation time observed via plate counts was 11.1 minutes for biotype 1 under the most optimal experimental conditions (pH 7,

temperature 37°C, salinity between 10-30ppt). The generation time estimated by qPCR measurements of total cells under the same conditions was 17.2 minutes.

All three variables had a significant effect on the growth rates of all *V. vulnificus* biotypes. Analyses were done using CFU/ml and logistic growth rate calculations. Temperature affected the growth of biotype 1 strain CMCP6 more than any other variable, as it was responsible for 43.1% of total variation (Table 1). Salinity and pH accounted for 12.2 and 4.3% of total variation respectively. Interactions among all the variables, except between temperature and pH were also significant, representing 19.0% of total variation when combined. Salinity was the most significant variable for biotype 2 strain ATCC 33147, followed by temperature and pH and resulting in 34.0, 24.8 and 1.5% of total variation of the growth rates respectively. Interactions among variables were also significant yielding 26.0% of total variation. For strain 302/99 (biotype 3), the levels of importance of all variables were analogous to biotype 2 – salinity>temperature>pH – resulting in 38.9, 26.8, and 0.5% of total variation respectively. Interactions of all variables, except between pH and temperature, were significant and responsible for 15.5% of total variation. (Table1).

CMCP6 (biotype 1) was more tolerant to low salinity (5 ppt) and was less effected by salt concentrations than the other strains. We noted that at pH 8 and 37°C, the growth rate of CMCP6 was significantly higher at 10 and 15 ppt than at other salinities ($p<0.001$). In general, strains 33147 (biotype 2) and 302/99 (biotype 3) behaved similarly, showing little response to salinity over the range of 15-40 ppt, but growing more slowly at 10 ppt under certain conditions. The growth rates of all three biotypes of *V. vulnificus* were greatest at 37°C and pH 7. However, we observed that the growth rates of biotypes 2 and 3 at 15ppt and pH7 were unaffected by temperature.

In summary the growth rates of all three *V. vulnificus* biotypes were primarily affected by temperature and salinity. The fastest growth rates were always observed at 37°C and pH7; however, responses to salinity were more variable by strain and were affected by other parameters. Biotype 1 was more tolerant to low salinities, and grew substantially faster than biotypes 2 and 3. It is, therefore, not surprising that biotype 1 strains represent the majority of human pathogens, as the human body represents an assembly of conditions beneficial for their growth: stable temperature of 37°C, blood salinity 8.5 ppt, and blood pH level 7.

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Table 1. Statistical analysis of logistic growth rates for representative biotype 1, 2 and 3 strains. Growth rates were determined by measuring CFU/ml (A) or total cells/ml by qPCR (B). Three-way ANOVA was used to test the effects of pH, temperature and salinity on growth rates of each strain. (*refers to interaction among the variables)

A	CMCP6-Biotype 1		ATCC 33147-Biotype 2		302/99-Biotype 3	
Physical Variable	**P**	**% Variation**	**P**	**% Variation**	**P**	**% Variation**
pH	<0.0001	4.3	<0.0001	1.5	0.020	0.6
Temperature	<0.0001	43.1	<0.0001	24.9	<0.0001	26.8
Salinity	<0.0001	12.2	<0.0001	34.0	<0.0001	38.9
Interaction						
pH*Temperature	0.122	0.5	0.0020	1.0	0.065	0.6
pH*Salinity	<0.0001	3.7	<0.0001	2.8	0.011	2.0
Temperature*Salinity	<0.0001	6.6	<0.0001	11.6	<0.0001	6.2
pH*Temperature*Salinity	<0.0001	8.7	<0.0001	10.6	<0.0001	7.4
Contribution of all physical variables to total variation	**x**	**77.0**	**x**	**84.2**	**x**	**79.2**

B	CMCP6-Biotype 1		ATCC 33147-Biotype 2		302/99-Biotype 3	
Physical Variable	**P**	**% Variation**	**P**	**% Variation**	**P**	**% Variation**
pH	0.006	1.0	0.029	0.3	<0.0001	5.1
Temperature	<0.0001	40.4	<0.0001	23.4	<0.0001	26.5
Salinity	<0.0001	12.2	<0.0001	42.6	<0.0001	42.1
Interaction						
pH*Temperature	0.074	0.7	.0546	0.1	<0.0001	1.2
pH*Salinity	<0.0001	5.7	<0.0001	7.7	0.002	1.7
Temperature*Salinity	<0.0001	10.2	<0.0001	11.4	<0.0001	3.9
pH*Temperature*Salinity	<0.0001	9.4	<0.0001	11.0	<0.0001	7.8
Contribution of all physical variables to total variation	**x**	**77.3**	**x**	**87.1**	**x**	**85.4**