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Thermal-Death Times of Opaque and Translucent Morphotypes of *Vibrio vulnificus*

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Thermal-death times were determined for *Vibrio vulnificus* strains with different morphotypes. Opaque strains showed higher D values (times required to reduce the viable population of a given strain by 90%) than translucent strains. Z values (absolute values of the temperature required to reduce 1 log scale of D values) were also significantly higher in opaque morphotypes (2.4 to 2.5°C) than in translucent ones (1.7 to 2.1°C). These results indicate that the morphotype is related to the organism's susceptibility to heat.

It has been demonstrated that *Vibrio vulnificus* has two distinct colony morphotypes, opaque and translucent (2, 14). The opaque strains are encapsulated, whereas translucent variants have reduced or no capsular materials (2, 9, 14). The opaque morphology has been correlated with decreased hydrophobicity, the ability to utilize transferrin-bound iron for growth, and high levels of virulence (5, 12, 14, 16). In addition, loss of capsule is accompanied by a decrease in virulence in mice and in resistance to the bactericidal activity of serum (16). Opaque strains have been reported to shift spontaneously to translucent variants at a frequency of about 10^{-4} (2, 14).

The spontaneous phase shifting of *V. vulnificus* in the laboratory strongly supports the possibility that the pathogen exists as a mixture of both morphotypes in nature (12). In comparison to the substantial body of literature differentiating the phenotypic characteristics in the context of pathogenicity or virulence, the relationship of morphotypes with physiological properties such as survivability in extreme temperatures has not been well described. Ingestion of undercooked seafoods is still one of the major sources of outbreaks of *V. vulnificus* infection (4, 8), and survival of the pathogen during thermal processing is of special concern for the safety of seafoods. Information about the susceptibility of virulent opaque and avirulent translucent strains to heat will provide valuable guidelines for designing thermal processing strategies to eliminate the pathogen from seafoods. In the present study, by examination of *V. vulnificus* strains having either opaque or translucent colonies, the susceptibilities to heat of the two morphotypes were compared.

Bacterial strains and culture conditions. A total of eight *V. vulnificus* strains were used throughout this study and are listed in Table 1. Five of the strains, SC95-02, SC95-07, SC95-16, SC95-33, and SC96-17, are either environmental or clinical isolates obtained from the coastal area of Chonnam Province, Korea, or from patients of Chonnam National University Hospital (Kwangju, Korea). They were confirmed as *V. vulnificus* through biochemical tests (13) with the API 20E kit (BioMerieux SA, Marcy l'Etoile, France) and specific amplification of the *vvhA* gene (15) by PCR (6). Three phase variants of *V. vulnificus*, MO6-24/O, MO6-24/T, and CVD752, were kindly provided by J. Glenn Morris, Jr., University of Maryland, Baltimore. Strains MO6-24/T and CVD752 are isogenic translucent mutants of the MO6-24/O opaque strain derived through spontaneous or insertional mutation with Tn*phoA*, respectively (16).

All *V. vulnificus* strains were grown in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, Mich.) or BHI agar supplemented with 2% salt (6). For strain CVD752, kanamycin (50 μg/ml) was added to the culture.

Rate of phase variation. To observe phase variations of the strains, a colony with a particular morphotype was inoculated into BHI broth. The culture was agitated in a 30°C shaker at 220 rpm for 10 h, and then cells were spread onto a heart infusion (Difco Laboratories) agar plate that was reported to provide an excellent contrast for the two colony types (12). The rates of colony phase variations were determined as the number of colonies showing the opposite morphotype per total number of colonies.

The morphotype shifts of opaque strains MO6-24/O, SC95-16, SC95-33, and SC96-17 to the translucent morphotype were at rates ranging from 10^{-5} to 10^{-4}. However, a shift of translucent strains SC95-02, SC95-07, MO6-24/T, and CVD752 to the opaque morphotype was not observed (Table 1). This result indicated that the phase variation from an opaque to a translucent type might be unidirectional, as suggested by previous studies (12, 16). Alternatively, the shifting rates of those translucent strains to the opaque type might simply be below the limit of our observation.

In either case, the rates of these phase variations appeared to be lower than those observed by other investigators (14, 16). Strain MO6-24 O/T was reported to shift to the opposite morphotype at rates of 10^{-4} and even 10^{-3} (14). This phase shift was reversible, indicating that capsular expression may be controlled by a reversible genetic rearrangement (14). The discrepancy in the rate of phase shifting among our and other investigators' results could partly be attributed to different culture conditions. Our culture conditions seem to be more optimal for phase stabilization than those used by other investigators. The report that the rates of phase shifting were varied when cells were grown under different conditions or with different stresses supports this possibility (2).

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TABLE 1. Reversion rate and morphological characteristics of *V. vulnificus* strains used in this study

Strain and morphotype	Reversion rate[a]	Source or reference
Opaque		
MO6-24/O	8.9×10^{-5}	14
SC96-17	9.1×10^{-5}	Seafood
SC95-16	3.8×10^{-5}	Seawater
SC95-33	6.7×10^{-5}	Clinical
Translucent		
MO6-24/T	$<1.9 \times 10^{-5}$	14
CVD752	$<8.3 \times 10^{-5}$	14
SC95-02	$<3.5 \times 10^{-5}$	Clinical
SC95-07	$<5.9 \times 10^{-5}$	Clinical

[a] Rate of shift to the opposite morphotype.

Calculation of D values and Z values. For the determination of thermal-death times, 5-ml aliquots of BHI broth were dispensed in 15-ml polypropylene culture tubes (Sarstedt, Germany) and adjusted to four different target temperatures (45, 47, 49, or 51°C) prior to incoculation. They were inoculated with each strain to an initial density of approximately 10^8 CFU/ml and incubated in a shaking water bath. For viable cell counting, samples (100 μl/each tube) were removed from the cultures at designated time intervals. The samples were immediately diluted with ice-cooled artificial seawater (10) and spread plated on BHI agar plates.

The numbers of surviving bacteria (log CFU per milliliter) from heat treatment were plotted against time for each temperature. D values, the time required to reduce the viable population of a given strain by 90%, were calculated from the best-fit line of the survival curves obtained through linear regression analysis. For linear regression analysis, at least five datum points were adopted. Each datum point was determined by averaging the results from at least three trials. D values and the correlation coefficient (r^2) of each survival curve were determined with Matlab software (Math Works, Inc., Natick, Mass.) (11). Z values were determined from the best-fit line obtained through plotting log D values against temperatures (Matlab software), and are expressed as the absolute value of temperature required to reduce 1 log scale of D values. The statistical significance of the difference between the two morphotypes was evaluated with Student's unpaired *t* test. Significance was accepted at the 0.05 level of probability.

TABLE 2. D values and Z values of *V. vulnificus* strains of opaque and translucent morphotypes

Strain and morphotype	D value (min) at[a]				Z value (°C)
	45°C	47°C	49°C	51°C	
Opaque					
MO6-24/O	43.48	3.55	0.48	0.20	2.5
SC96-17	47.62	3.37	0.59	0.19	2.5
SC95-16	46.30	3.86	0.59	0.18	2.5
SC95-33	55.60	3.40	0.53	0.19	2.4
Translucent					
MO6-24/T	39.30	3.31	0.47	0.17	2.1
CVD752	40.94	3.08	0.39	0.16	1.7
SC95-02	42.08	3.20	0.39	0.19	2.0
SC95-07	40.00	3.53	0.49	0.19	2.1

[a] D values were calculated from each linear regression survival curve.

TABLE 3. Comparison of D values between opaque and translucent morphotypes

Temp (°C)	D value (mean ± SE) for morphotype:		*P* value[a]
	Opaque ($n = 4$)	Translucent ($n = 4$)	
45	48.25 ± 2.60	40.58 ± 0.60	0.028
47	3.55 ± 0.11	3.28 ± 0.10	0.122
49	0.55 ± 0.03	0.44 ± 0.03	0.024
51	0.19 ± 0.00	0.18 ± 0.01	0.194

[a] Statistical significance of the difference between the two morphotypes was evaluated with Student's unpaired *t* test. Significance was accepted at $P < 0.05$.

Thermal-death times. Correlation coefficients of the linear regression survival curves ranged between 0.803 and 0.991. The opaque strains appeared to be more resistant to the heat treatment than the translucent strains. The D and Z values of the strains tested are shown in Table 2. Mean D values for opaque strains were higher than those for translucent ones at all of the heat treatment temperatures (Table 3). Statistically significant differences were observed at 45 and 49°C.

Z values were also higher in opaque strains than in translucent strains. The mean Z value was significantly higher in opaque strains than in translucent strains ($P < 0.005$ [Fig. 1]). This result indicates that inactivation of translucent strains is more profoundly potentiated by increasing temperature than that of opaque strains (7). The Z values of either opaque or translucent *V. vulnificus* strains are significantly lower than those of other food-borne bacteria, including *Escherichia coli* O157:H7, *Salmonella typhimurium*, and *Listeria monocytogenes*. The Z values for these pathogens have been reported to be around 4 to ~5°C (1, 3). The results presented above suggest that heating is a relatively effective method for eradicating *V. vulnificus* from seafoods and also indicate that changes of the internal temperature of even as little as 1 or 2°C can profoundly influence the outcome of heat eradiction of *V. vulnificus* contaminating seafoods.

From these results, it is evident that opaque morphotypes are more resistant to heat than translucent types, suggesting

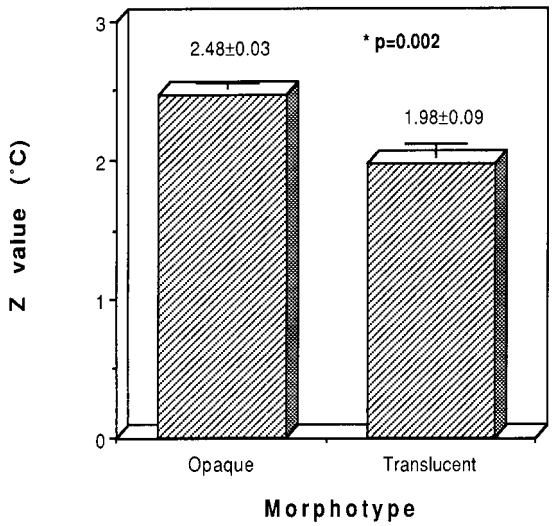

FIG. 1. Comparison of mean Z values between opaque and translucent morphotypes. Error bars represent standard errors of the mean. The statistical significance of the difference between the two morphotypes was evaluated with Student's unpaired *t* test. Significance was accepted at $P < 0.05$.

that capsular material might serve as the cellular insulator for *V. vulnificus*. Variance in the Z values was more prominent in the translucent group. Among the translucent strains tested, SC95-02, SC95-07, and MO6-24/T appeared to have distinctively higher Z values than those of CVD752 (Table 1). The correlation between the quantity of capsular material and heat resistance was not investigated in the present study. Either chemical measurement of capsular polysaccharide or morphometric analysis of ruthenium red-stained electron micrographs in correlation with heat resistance will provide, we believe, more objective information. An indirect, but plausible, explanation for the role of capsular material in the heat resistance could be deduced from comparison of the Z values of isogenic variants MO6-24/T and CVD752. Spontaneous translucent variant MO6-24/T had a higher Z value than transposon insertional mutant CVD752. Wright et al. showed by electron microscopic examination that MO6-24/T still had a reduced amount of capsule compared with that of the wild-type MO6-24/O strain, while CVD752 was completely devoid of capsular polysaccharide (14). This difference in the amount of capsule might explain the difference in Z values between the two isogenic variants.

Increasing the inactivation temperature to 51°C resulted in a significant decrease in the D value of *V. vulnificus* in both morphotypes. As the temperature increased from 45°C to 51°C, the difference between the D values of the two morphotypes became minute (Tables 2 and 3). Since *V. vulnificus* is innately more susceptible to heat than the other food-borne pathogens stated above, the insulating effect of capsular material seems easily overcome by increasing the temperature above a certain critical point. This suggests that if we can determine the critical temperature, we can guarantee reliable elimination of viable *V. vulnificus* organisms from seafoods irrespective of colony morphotypes.

Raw seafoods contaminated with *V. vulnificus* remain a major public health problem. In an effort to reduce health risks, various commercial methods have been employed to control pathogens in food. Although the application of heat has been recognized as an effective measure for inactivating bacteria involved in food poisoning, heating is complicated by the deterioration of nutritional or sensory value (7). Establishment of safe guidelines for heat processing of seafood is mandatory for the prevention of *V. vulnificus* septicemia. The present study clearly shows that virulent opaque strains are more resistant than translucent strains to heat treatment and that *V. vulnificus* is more susceptible to heat than other food-borne pathogens are. We hope our data can provide basic information for designing an effective thermal process that ensures the highest quality of *V. vulnificus*-free seafoods.

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