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Physiology and Ecology of Bacteriophages of the Marine Bacterium *Beneckea natriegens*: Salinity[1]

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The effects of variation in ionic levels on the stability and replication of two bacteriophages (nt-1 and nt-6) host specific for the marine bacterium *Beneckea natriegens* were examined. Monovalent cations influenced the adsorption of the nt-1 but not the nt-6 phage; however, one-step growth studies showed that NaCl was required for replication of both phage. The NaCl optimum for nt-1 production was 0.25 M NaCl, the same as the growth optimum for *B. natriegens*. However, the optimum for nt-6 production was 0.16 M NaCl. These NaCl optima for host and phage are at estuarine rather than oceanic levels. The nt-1 phage was better suited to replicate at NaCl levels typical of higher salinity areas (18–35‰) and the nt-6 phage was better suited to replicate at lower salinities (5–18‰). The nt phage were more resistant to low NaCl levels than their host bacterium and appeared limited to marine waters by the lower survival salinity of *B. natriegens* coupled with phage inactivation processes occurring in natural estuarine waters.

Beneckea natriegens (4, 5) is a marine bacterium originally isolated from salt marsh mud (23) and grows extremely well in the laboratory (9, 22). The metabolic (7, 10–12, 25) and respiratory capabilities of *B. natriegens* (18, 35–38), as well as its characteristic marine requirement for sodium ions for protein synthesis and growth, have been studied in detail (23, 24, 27–30, 34). Bacteriophages active against *B. natriegens* are widely distributed in coastal salt marshes, where they appear to be abundant and can be easily isolated (40). These phages are limited to marine waters, and in estuaries their distribution appears to be salinity dependent (40).

B. natriegens and its phages provide a representative marine system in which the influence of environmental factors on the physiology of these organisms can be determined in the laboratory to provide a basis for explaining their distribution in the field. In this study the effects of NaCl levels (most abundant salt in seawater) on *B. natriegens* phage stability and replication were examined as a possible means of determining how salinity affects the distribution of these phages in marine waters.

(Portions of this work were presented at the 74th Annual Meeting of the American Society for Microbiology, May 1974. This work is part of a dissertation submitted to the School of Marine Science of the College of William and Mary in partial fulfillment of the requirements for the Ph.D. degree.)

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MATERIALS AND METHODS

Isolation and enrichment. The methods of isolating bacteriophages of *B. natriegens* and preparation of high-titer stocks were previously described (40). Single phage isolates were obtained by stabbing plaques with a sterile wire dipped in sterile medium and inoculating enrichment cultures. The purity of these isolates was checked by electron microscopy.

Host bacterium. *B. natriegens* was obtained from the American Type Culture Collection (no. 14048). The bacteria were grown on a rotary shaker at 150 rpm at 27 C. Cell density was determined by measuring absorbance at 580 nm using a Spectronic 20 spectrophotometer (Bausch and Lomb), and conversions to cell numbers were made using a standard curve. Phage titers were determined by plaque assay using the agar layer technique (2). Unless otherwise stated, the underlayer consisted of nutrient agar (Difco) made with 15 to 18‰ aged estuarine water (EW) collected from the York River estuary in Virginia. The overlay consisted of 0.7% agar (Difco) made with EW.

Growth media. Broth media containing the following nutrients (in grams per liter) were used: nutrient broth (Difco), 10.0; peptone (Difco), 5.0; and yeast extract (Difco), 2.5. These nutrients were added to either EW or to a four-salts solution, which contained 0.16 M NaCl, 3.8 mM KCl, 0.018 M $MgSO_4 \cdot 7H_2O$, and 3.8 mM $CaCl_2 \cdot 2H_2O$. These salt levels approximated the values found in EW of 14‰

(33). Atomic adsorption spectrophotometry showed 6.4 μg of Na^+ per ml in the distilled water used and 64 μg of Na^+ per ml when nutrients were added.

In experiments involving variation of the NaCl levels, only the amount of NaCl was changed and the other salts were kept at the same levels. The highest NaCl value tested, 0.41 M, represented oceanic salinity, 35‰. For most experiments the media were sterilized by autoclaving; however, in experiments where osmotica were used it was necessary to filter sterilize (0.22-μm pore size; Millipore Corp.) the media used, including the controls. The osmotica, either mannose (not utilized by *B. natriegens*) or KCl, were added to low NaCl media to raise the osmolarity to that of the 0.16 M NaCl four-salts medium (430 mosmol). Osmolarity was checked in all cases using an Osmette osmometer (Precision Systems).

Host range. Bacterial cultures were kindly provided by R. R. Colwell and C. W. Vermeulen. The bacteria were grown in four-salts nutrient medium (4SN), and the agar used for platings and overlays was also made using 4SN medium. Lawns of the various bacteria were spotted with high-titer stocks of the phages and then incubated at 27 and 37 C and checked for clearing at 24 and 48 h.

Survival experiments. Phage dilutions were made in 100 ml of the desired medium and stored at 20 C. Periodically the titer was determined by plaque assay.

Plaque formation. The plaque-forming ability of the phages was determined by plaque assay using 4SN medium of various NaCl concentrations. The same medium was used for growth of the bacteria, dilution of phage, and preparation of plates and overlays. Noble agar (Difco) was used in these experiments to reduce contamination with salts. Plaques were counted at 24 and 48 h after incubation at 27 C.

Adsorption. Host bacteria were grown to a density of 2×10^8 cells/ml in 4SN medium (0.16 M NaCl) and then centrifuged (10,000 $\times$ *g*, 5 min, 4 C) and washed with 4SN medium of the desired NaCl level. Aliquots were then added to 50-ml flasks containing 10 ml of 4SN medium to give a final cell concentration of 10^8 cells/ml. Prior to addition of the host bacteria, phage were added to the flasks at a multiplicity of infection of 1, with samples removed for plaque assay. After addition of bacteria, the flasks were incubated for 20 min at 27 C on a shaker at 100 rpm. The contents of the flasks were then centrifuged (10,000 $\times$ *g*, 5 min, 4 C), and the supernatants were sampled and titered by plaque assay.

One-step growth. The procedure for one-step growth experiments was similar to that of Kelln and Warren (16). The host bacteria were grown to a density of 2×10^8 cells/ml in 4SN medium of the desired NaCl level. Phage were added at a multiplicity of infection of 0.05 to 0.1 and incubated for 20 min at 27 C at 100 rpm. A 1.5-ml aliquot was then removed from the flask and filtered (0.45-μm pore size; Millipore Corp.), and then 0.5 ml of fresh medium was passed through the filter (filtration effectively eliminated unadsorbed phage, thus compensating for the low adsorption observed for phage nt-6 [Fig. 3]). The filter was then placed in 50 ml of 4SN medium and agitated to resuspend the cells. A 0.1-ml aliquot of resuspended cells was then placed in 99.9 ml of fresh medium and returned to the shaker at 125 rpm. Where necessary, a second dilution was made into another flask. The flasks were kept on the shaker, and periodically samples were removed for plaque assay. Unadsorbed phage were determined by assay of chloroform-treated duplicate samples removed at 25 to 30 min postinfection. For each condition tested, at least three growth experiments were conducted and results reported are mean values.

RESULTS

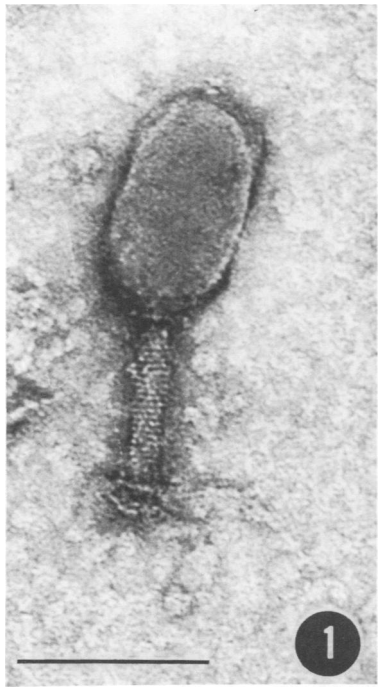
The nt-1 phage has a prolate, 120- by 70-nm head and a 110-nm contractile tail (Fig. 1). The nt-1 phage would therefore be grouped among other T-even-like phages as a type A2 myovirus (1). The nt-6 phage had an isometric 60-nm diameter head and a short 40-nm tail with tail appendages (Fig. 2). The phage resembled *Salmonella* phage P22 and would be classified as a type C1 phage (1).

Specific ionic requirements for phage viability have been proposed as a possible means by which to distinguish marine phage from nonmarine phage (32). The results of studies on inactivation of nt phage in various solutions did not support this hypothesis. The nt-6 phage titer decreased in pond water and distilled water but not at low NaCl levels in 4SN. The nt-1 phage was stable in all solutions including distilled water (Table 1).

Studies have shown that titers decrease when marine phages are kept in untreated seawater; however, this phage inactivation can be prevented by sterilization of the seawater (3). The nt phage titers decreased when they were incubated in freshly collected EW, but the titers remained stable in EW that had been sterilized by autoclaving or filtration (Table 1).

Early studies suggested that marine phage might be more susceptible to inactivation by elevated temperatures than nonmarine phage (32). The nt-1 phage was stable at temperatures up to 37 C, showed a slow titer decrease (50% after 20 days) at 50 C, and was rapidly inactivated at 60 C (Table 2). The nt-6 phage was more susceptible to thermal inactivation, showing titer decreases at 37 C and rapid inactivation at 50 and 60 C (Table 2). Thermal inactivation occurs at about the same rate in many nonmarine phage, especially after heating in salt solutions, which apparently increases their susceptibility to inactivation (2).

The nt-1 and nt-6 phage were host specific for *B. natriegens* and did not replicate on any of the alternate marine and nonmarine host bacteria tested (Table 3). *B. natriegens* has been shown



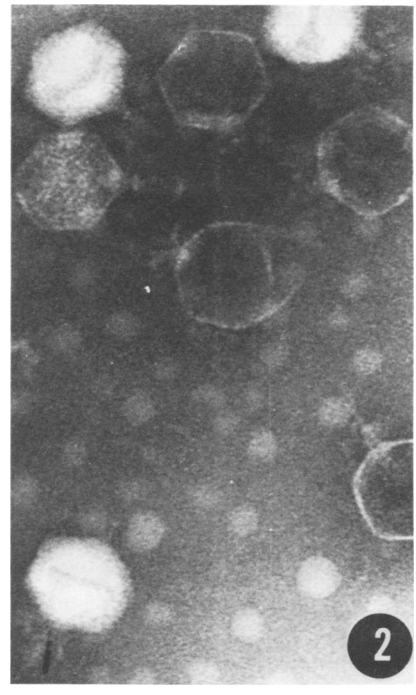


FIG. 1. *Phage nt-1 negatively stained with 2% aqueous uranyl acetate. Bar, 100 nm.*

FIG. 2. *Phage nt-6 negatively stained with 1.5% phosphotungstic acid (pH 7.2). Magnification is the same as Fig. 1.*

TABLE 1. *Phage survival after incubation in various media at 20 C*

Solution	PFU[a] of nt-1 phage remaining (%)				PFU of nt-6 phage remaining (%)			
	10 days	30 days	60 days	120 days	10 days	30 days	60 days	120 days
4SN (0.16 M NaCl)	100	100	100	100	100	100	100	100
4SN (0.06 M NaCl)	100	100	100	100	100	100	100	100
Pond water	100	100	100	100	85	75	50	45
Distilled water	100	100	100	100	50	5	4×10^{-4}	4×10^{-4}
Autoclaved EW	100	100	100	100	100	100	100	NO[b]
Filtered EW	100	100	100	100	100	100	100	NO
Untreated EW	14	12	11	NO	38	1	0.1	NO

[a] PFU, Plaque-forming units.
[b] NO, No observation made.

TABLE 2. *Survival of phage diluted in 4SN medium (0.16 M NaCl) after incubation at varius temperatures*

Temp (C)	PFU[a] of nt-1 phage remaining (%)				PFU of nt-6 phage remaining (%)			
	1 h	2 h	10 h	20 h	1 h	2 h	10 h	20 h
5	100	100	100	100	100	100	100	100
27	100	100	100	100	100	100	100	100
37	100	100	100	100	100	70	50	49
50	100	92	67	50	50	49	4	0.4
60	8.3×10^{-4}	TL[b]	TL	TL	0.3	0.03	TL	TL

[a] PFU, Plaque-forming units.
[b] TL, Numbers too low for detection.

TABLE 3. *Host range of nt-1 and nt-6 bacteriophages*

Bacterium	Lysis	
	nt-1	nt-6
Bacillus cereus	–	–
B. subtilis	–	–
Micrococcus luteus	–	–
Staphylococcus aureus	–	–
Sarcinia lutea	–	–
Escherichia coli B	–	–
E. fruendii	–	–
Klebsiella pneumoniae	–	–
Aerobacter aerogenes	–	–
Citrobacter freundii	–	–
Proteus mirabilis	–	–
Flavobacterium capsulatum	–	–
Pseudomonas aeruginosa	–	–
P. fluorescens	–	–
P. bathycetes	–	–
Vibrio marinus	–	–
V. parahaemolyticus (FC1011)	–	–
V. parahaemolyticus (SAK 3)	–	–
V. parahaemolyticus (SAK 4)	–	–
V. parahaemolyticus (341)	–	–
V. alginolyticus (157-70)	–	–
V. alginolyticus (163-70)	–	–
Beneckea natriegens	+	+

to have a specific requirement for the sodium ion for protein synthesis (34). In 4SN medium a medium of 0.06 M NaCl was required for growth of *B. natriegens* and optimal growth occurred at 0.25 M NaCl. These are the same lower limit and optimum reported previously by Payne (24). Since sodium ions are essential for *B. natriegens,* studies were conducted to determine the effects of various NaCl levels on replication of the nt phage. The nt phage were able to form plaques at values of 0.06 M NaCl or greater, suggesting that phage replication can occur at all NaCl values at which the host bacterium can survive.

The first step in the phage replicative process is adsorption of the phage to receptor sites on the outer membrane of the host bacterium. Although the nt-1 and nt-6 phage have the same host, the adsorption characteristics of each phage were quite different. Phage nt-1 adsorption was low at 0.06 M NaCl (8%) but rose rapidly to 84% adsorbed as NaCl levels were increased to 0.16 and 0.25 M NaCl and then decreased slightly to 82% at 0.41 M NaCl (Fig. 3). Use of mannose, a neutral osmoticum, did not improve phage nt-1 adsorption; however, KCl, an ionic osmoticum, completely compensated for NaCl (Table 4). Thus nt-1 phage adsorption was dependent on the level of monovalent cations in the medium. In contrast, adsorption of phage nt-6, which was low (26%) at all

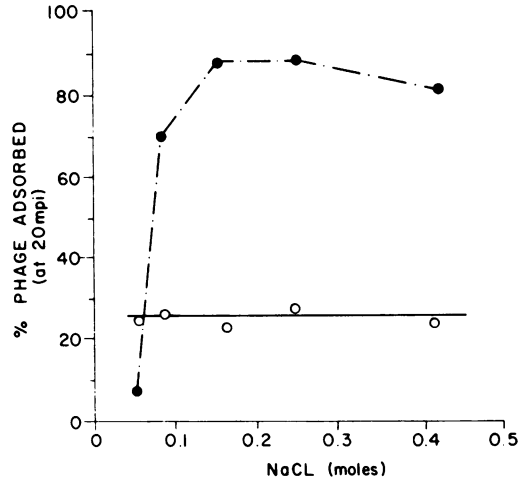


FIG. 3. *Percentage of nt-1 (●) and nt-6 (○) phages adsorbed after 20 min in 4SN media of various NaCl concentrations at 27 C.*

TABLE 4. *Phage nt-1 adsorption in 4SN media and 4SN media supplemented with osmotica*[a]

NaCl (mol)	nt-1 phage adsorbed after 20 min (%)		
	Control	Mannose added	KCl added
0.06	24	30	84
0.08	47	41	85
0.16	82	NO	NO

[a] Either KCl or mannose was added to raise osmolarity of 0.06 or 0.08 M NaCl 4SN media to that of 0.16 M NaCl medium (430 mosmol). The media were filter sterilized. NO, No observation made.

NaCl levels tested, appeared to be independent of ionic levels (Fig. 3).

The one-step growth technique provided a means of quantitation of the effects of variations in ionic levels on phage replication. Replication of both phage varied with changes in the NaCl concentration, and the effects on replication were reflected in changes in both latent period and burst size (Tables 5 and 6). Since burst size and latent period varied with the NaCl levels, in comparing experiments, it was convenient to use the rate of phage synthesis (plaque-forming units per cell per minute of latent period) obtained by dividing the burst size by the latent period for each one-step growth experiment (Tables 5 and 6; Fig. 4). The effect of varied NaCl levels was different for each of the phages, and each had a different NaCl optimum for phage synthesis. The synthesis of nt-1 phage in response to various NaCl levels paralleled the growth of its host bacte-

TABLE 5. *Effects of NaCl levels on nt-1 phage production*[a]

NaCl (mol)	Latent period (min)	Burst size (PFU/cell)	Rate (PFU/cell/min of latent period)
0.06	90	12	0.13
0.08	90	112	1.24
0.16	60	321	5.35
0.20	53	413	7.8
0.25	45	520	11.60
0.33	50	522	10.45
0.41	50	511	10.20

[a] These one-step growth experiments were conducted at 27 C in autoclaved 4SN media of varied NaCl concentrations. PFU, Plaque-forming units.

TABLE 6. *Effects of NaCl levels on nt-6 phage production*[a]

NaCl (mol)	Latent period (min)	Burst size (PFU/cell)	Rate (PFU/cell/min of latent period)
0.06	90	300	3.33
0.08	67-75	605	8.52
0.13	60	609	10.20
0.16	55	610	11.50
0.20	57	552	9.80
0.25	60	499	8.32
0.33	60	311	5.20
0.41	60	311	5.20

[a] These one-step growth experiments were conducted at 27 C in autoclaved 4SN media of varied NaCl concentrations. PFU, Plaque-forming units.

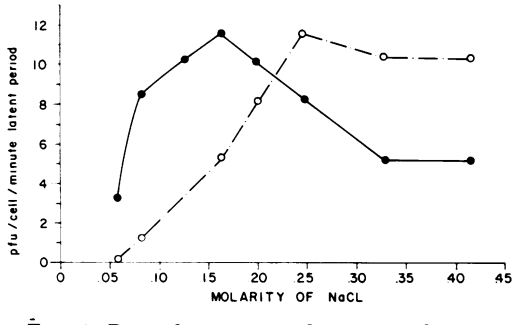


FIG. 4. *Rate of nt-1 (○) and nt-6 (●) phage production (plaque-forming units per cell per minutes of latent period) based on one-step growth experiments at 27 C in 4SN media of various NaCl concentrations.*

rium at various NaCl levels. The rate of nt-1 synthesis was lowest at 0.06 M NaCl and increased as NaCl levels increased, reaching a maximum rate at 0.25 M NaCl. At NaCl levels above this maximum, minor decreases occurred in the rate of nt-1 synthesis (Fig. 4). Phage nt-6 production was maximal at 0.16 NaCl, a value less than optimal for growth of its host or the nt-1 phage. Of particular interest is the observation that nt-6 phage production was greater than nt-1 phage production at values between 0.06 and 0.20 M NaCl, and at higher NaCl values (0.22 to 0.41 M NaCl) nt-1 production was greater than nt-6 phage synthesis (Fig. 4).

For both phages, osmotica only partially compensated for NaCl in respect to phage production. The ionic osmoticum, KCl, was more effective then the neutral osmoticum, mannose (Tables 7 and 8). A similar compensation effect of K^+ ions for Na^+ ions has been observed in growth of the host bacterium, *B. natriegens* (34).

DISCUSSION

Obligately marine phages occur only in estuarine and oceanic environments, which implies that they can survive and replicate at "in situ" conditions of their marine habitat. Replication also implies the presence of a marine host bacterium with characteristic ionic requirements, especially a specific need for Na^+ ions at marine levels (19, 20). Similar ionic requirements have

TABLE 7. *Phage nt-1 phage production in 4SN media and 4SN media supplemented with osmotica*[a]

Medium	Latent period (min)	Burst size (PFU/cell)	Rate of phage synthesis (PFU/cell/min of latent period)
0.16 M NaCl	45	750	16.7
0.08 M NaCl	80	40	0.5
0.08 M NaCl + mannose	85	95	1.1
0.08 M NaCl + KCl	60	430	7.2

[a] Either mannose or KCl was added to 0.08 M NaCl 4SN media to raise osmolarity to a level of 0.16 M NaCl 4SN meidum. The media were filter sterilized. PFU, Plaque-forming units.

TABLE 8. *Phage nt-6 production in 4SN media and 4SN media supplemented with osmotica*[a]

Medium	Latent period (min)	Burst size (PFU/cell)	Rate of phage synthesis (PFU/cell/min of latent period)
0.16 M NaCl	60	1,655	27.6
0.06 M NaCl	90-95	520	5.6
0.06 M NaCl + mannose	70	712	10.2
0.06 M NaCl + KCl	60	1,000	16.7

[a] Either mannose or KCl was added to 0.06 M NaCl 4SN media to raise osmolarity to a level of 0.16 M NaCl 4SN medium. The media were filter sterilized. PFU, Plaque-forming units.

also been indicated for marine phage replication (13, 17, 32, 39).

The nt-1 and nt-6 phages were obligately marine in that they could only be isolated from marine waters (40); they were host specific for *B. natriegens*, a marine bacterium and could replicate at conditions approximating those of their habitat; and replication was clearly NaCl dependent.

Study of marine bacteriophages has shown that, contrary to earlier suggestions, these phage have physical characteristics such as morphology, ionic requirements for stability, and thermal inactivation that are similar to phage of nonmarine origin (6, 13, 15, 17, 31). This same observation was made for the nt phage (Fig. 1 and 2; Tables 1 and 2). This similarity in the physical characteristics of phage from varied environments likely reflects the resistant nature and resultant reduced susceptibility of extracellular virions to selection by environmental factors. Physical characteristics of phage therefore seem useful for relating similar phage types rather than distinguishing phage from different environments.

During replication, the phage-infected cell represents a stage in the phage life cycle that is most susceptible to the selective influences of environmental factors. As a result, the criteria that have proved most useful in distinguishing marine from nonmarine phages were the physiological characteristics of phage replication, especially with respect to factors important in their environment (14, 17, 39).

B. natriegens phage replication was possible at NaCl levels of 0.06 M or greater, and phage production varied with the NaCl level of the growth medium. The nt-1 phage production was maximal at 0.25 M NaCl, the host's growth optimum. In contrast, nt-6 phage production was greatest at 0.16 M NaCl, a value less than the host cell optimum (Fig. 3; Tables 5 and 6). Expression of the phage genome after infection can change the physiological properties of the host cell and can therefore effect the ecology of the phage. The differences in synthetic abilities of nt-1 and nt-6 phage-infected cells at various NaCl levels raises the possibility of natural selection based on salinity, since these phage compete for the same host cells in estuarine waters characterized by fluctuating salinities (41). The nt-1 phages were better able to replicate at NaCl levels typical of higher salinity areas (18 to 35‰), whereas the nt-6 phages replicated better at NaCl levels typical of lower salinity areas (5 to 18‰; Fig. 3). Adsorption of nt-6 phage was lower than nt-1 adsorption at all NaCl levels, except those typical of low salinities (~5‰) where nt-1 phage adsorption was severely reduced (Table 4). Thus the selective advantage of phage nt-6 at lower salinities is further enhanced by the effect of ionic levels on nt-1 phage adsorption. Field data showed that the nt-6 phage was only isolated from lower salinity areas of the York River estuary (1 to 18‰), whereas the nt-1 phage was isolated from stations over the entire salinity range (40). This was consistent with the hypothesized selection on the basis of salinity; however, natural situations are highly complex and other factors also appeared to favor the wide distribution of the nt-1 phage (A. Zachary, manuscript in preparation).

In the York River estuary of Chesapeake Bay, nt phages were present in all areas with salinities of 8‰ or greater, their presence was sporadic in areas with salinities fluctuating between 1 and 7‰, and they were not present in freshwater areas (40). The transitional salinities between 1 and 7‰ indicated the lower survival salinity range for the phage. This salinity range includes 0.06 M NaCl, roughly the value expected at about 5‰ salinity, which was the lower NaCl limit for *B. natriegens* survival and phage plaque formation. The nt phage themselves were not inactivated by NaCl levels in this range and were viable at ionic conditions in which their host could not survive (Table 1). The absence of nt phage in low salinities therefore appears to be controlled by the lower NaCl limit of *B. natriegens* coupled with phage inactivation processes which occur in natural estuarine water. In areas of salinity too low for survival of *B. natriegens*, the phage cannot replicate, and their numbers would be reduced below detectable levels by natural inactivation processes. In areas where salinities are high enough for host cell survival, phage replication can occur, allowing replenishment of phage lost by inactivation and thus maintaining phage levels at detectable levels. Ahrens (3) reported that in the Kiel Bay estuary the numbers of *Agrobacterium* phage decreased as salinity fell below 8‰, the lower survival salinity of the host bacterium, and laboratory studies showed that the *Agrobacterium* phage were inactivated when agitated in untreated EW. The mechanism by which phage are limited to marine waters by salinity appears to be similar for these two different marine phage host systems.

B. natriegens and its phage were isolated from estuarine and coastal areas (23, 40), and both host bacterium and phage-infected cells showed maximum synthetic ability at NaCl levels typical of estuarine water (14 to 21‰) rather than oceanic waters (35‰). *Vibrio parahaemolyticus*, a marine bacterium taxonomi-

cally closely related to *B. natriegens* (4, 5), has a NaCl growth optimum quite similar to *B. natriegens*, i.e., at estuarine levels (26). Extensive study of the distribution of *V. parahaemolyticus* in the Chesapeake Bay and Atlantic coastal waters have shown that this bacterium is present only in estuarine waters (8). *Vibrio marinus*, a marine bacterium isolated from oceanic waters, has a NaCl growth optimum at levels representative of the higher salinities of its oceanic habitat (21). It appears that a relationship may exist between NaCl growth optima and environmental salinities, which reflects the selective influence of salinity on these marine bacteria. However, the ecology of marine organisms cannot be accounted for by the effects of salinity alone; other factors are involved, and their effects must also be considered.

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