Properties of Two Marine Bacteriophages

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ABSTRACT

CHEN, PETER K. (Georgetown University, Washington, D.C.), RONALD V. CITARELLA, OMAR SALAZAR, AND RITA R. COLWELL. Properties of two marine bacteriophages. J. Bacteriol. 91:1136-1139. 1966.—Various properties have been determined for two bacteriophages, NCMB 384 and 385, and their host, NCMB 397, a Cytophaga sp., isolated from the marine environment. The purified bacteriophages have been subjected to serological analysis, results of which indicate a high degree of relatedness. Purified, highly polymerized deoxyribonucleic acid (DNA) prepared from the host strain showed an overall base composition of 37 moles % guanine + cytosine (buoyant density of 1.696 g/cc). The bacteriophage DNA, in the native configuration, from NCMB 384 and 385 banded at 1.691 g/cc in a CsCl gradient and the denatured bacteriophage DNA demonstrated a bimodal peak. Stability tests of the bacteriophages in various buffers and diluents suggest a requirement for inorganic cations, most likely Na\(^+\) and Mg\(^{2+}\), for retention of viability.

Several investigators have reported the isolation of bacteriophages active against bacteria cultured from samples of coastal seawater (2, 21). However, Kriss and Rukina (6) and Spencer (14, 15) were able to demonstrate the presence of bacteriophages in waters beyond the littoral zone. Several genera and species of bacteria, isolated from the Black Sea and the North Sea, including Micrococcus, Bacillus, and Photobacterium species, served as host for the bacteriophages. Details of isolation, characterization, ecology, etc., of these bacteriophages are scanty, and only Spencer (16) presents detailed methods for the isolation of bacteriophages from seawater, as well as results of experiments carried out to determine the effect of temperature and ionic concentration on replication of the bacteriophages. The bacteriophages examined by Spencer were inactivated within 1 hr at 55 C and were capable of multiplication at temperatures of 0 to 2 C. Several bacteriophage isolates did not form plaques at temperatures above 30 C. An increased requirement for cations was also indicated (16).

More recently, Wiebe and Liston (Bacteriol. Proc., p. 38, 1964) reported a bacteriophage active against a marine pseudomonad; the bacteriophage survived and increased in number at 1,400 psi (90 atm) pressure. Temperature and salinity parameters for multiplication of the bacteriophage were also presented.

Little information exists, however, concerning the structure, biochemical nature, and growth characteristics of marine bacteriophages. The purpose of this study was to characterize biochemically and serologically two marine bacteriophages and their host strain. Eventually, it is hoped that by the accumulation of such data for marine bacteriophages, in general, their role in the marine environment may be elucidated.

MATERIALS AND METHODS

The bacteriophages employed in this study were NCMB 384 and NCMB 385 received from James M. Shewan, National Collection of Marine Bacteria, Torry Research Station, Aberdeen, Scotland. These strains were originally isolated by Spencer from the North Sea 10 miles from shore, and designated by him as P/SW1/a and P/SW1/b. Host strain NCMB 397 was tentatively classified as a Flavobacterium sp. by Spencer, but subsequent taxonomic work with this organism indicates that it is most probably a marine Cytophaga sp. (4).

Culture medium was prepared with a synthetic reconstituted sea salts mixture (Seven Seas Mix, Utility Chemical Co., Paterson, N.J.): 37.9 g of salts mixture per liter of distilled water. Temperature and growth studies of the host were performed with an artificial seawater consisting of, per liter of distilled water: NaCl, 2.4%; KCl, 0.07%; MgCl\(_2\)-6H\(_2\)O, 0.53%; and MgSO\(_4\)-7H\(_2\)O, 0.70%. Other components of the growth medium were yeast extract (Difco), 0.3%, and proteose-peptone (Difco), 1.0%. The synthetic sea salts medium was routinely autoclaved for 5 to 10 min at 15 psi at 120 C, cooled, filtered through glass wool to remove undissolved materials, and subse-
quenty distributed into test tubes or flasks for final autoclaving before use. Solid medium was prepared by addition of 1.5% (Difco) agar to the filtered broth.

The host culture, NCMB 397, was incubated at room temperature (22 to 25 C) overnight. The lysates were prepared from 24-hr seawater broth culture infected with bacteriophage at a multiplicity of 1.0. Bacteriophage stocks were prepared from the lysates after filtration through a Seitz filter, and were stored in the refrigerator. Plaque-forming units for bacteriophages 384 and 385 were assayed by the method of Adams (1), yielding ca. 10⁸ particles per milliliter.

**Purification of bacteriophage.** Three methods were employed to purify the bacteriophages from lysates and from the extracts of the completely lysed second layer: the ammonium sulfate precipitation (12), alcohol precipitation (19), and differential centrifugation (5) methods.

**Preparation of purified nucleic acid.** Deoxyribonucleic acid (DNA) was isolated from the host and the purified bacteriophage preparations by the method of Marmur (8). Ribonuclease and deoxyribonuclease were added to the purified and concentrated bacteriophage preparation prior to the extraction of the bacteriophage DNA to remove contaminating host nucleic acids.

**Determination of DNA base ratio.** Thermal denaturation-renaturation, i.e., helix-to-coil transition of the purified, highly polymerized host and bacteriophage DNA, were carried out according to the method of Marmur and Doty (10). Buoyant density measurements in cesium chloride (11) were also made. In the CsCl density-gradient centrifugations, approximately 1 to 2 μg of DNA was added to a 57% (w/w) stock solution of CsCl (American Potash & Chemical Corp., Los Angeles, Calif.), buffered to pH 8.0 with 0.015 M tris(hydroxymethyl)aminomethane (Tris) buffer, and the density of the sample was adjusted to 1.710 g/cm³, according to the linear relationship between refractive index and density. Ca. 0.75 ml of the final CsCl solution was placed in a cell containing a plastic Kel-F centrifuge tube and was centrifuged in a Spinco model E analytical ultracentrifuge at 44,770 rev/min at 25 C. After 20 hr of centrifugation, the banded DNA was photographed by use of ultraviolet-absorption optics. The photographs were traced with a Joyce-Loebl Mark III B doublebeam recording microdensitometer, and the buoyant densities of the DNA were calculated by reference to a standard DNA of known density which was included in each run (reference DNA = deuterated Escherichia coli, K-12 DNA).

Denaturation of bacteriophage and host DNA at 10 μg/ml was carried out by heating at 100 C for 10 min in 0.015 M NaCl + 0.0015 M Na-citrate, followed by quenching in an ice-water bath.

**Preparation of antisera, determination of K values, and one-step growth curves were performed according to the methods described by Adams (1).**

**Host specificity.** Host specificity, tested by using 1.0 ml of a 24-hr culture with 1.0 ml of lysate in the double-layer technique, was assayed with the following strains of marine bacteria: *Pseudomonas enalia* (Colwell and Sparks, in preparation), *P. fluorescens* strain PS 203, *Pseudomonas* spp. PS 653 and PS 612 (3), *Cytophaga* sp. strain B9 (7), *Vibrio* sp. strains MB 22 (17) and PS 523 (3), *P. phosphoreum* (15), *P. atlantica* (20), and *Pseudomonas* sp. NCMB 400. Eight strains of *Flavobacterium* sp. (ATCC 4385, 958, 8091, 4651, 46, 8315, 653, and 11497) were also tested.

**RESULTS**

The morphology of bacteriophage NCMB 385 has been determined and has been reported in detail elsewhere (18).

Host-specificity experiments showed that the two bacteriophages, NCMB 384 and 385, lysed only the original host NCMB 397. Other characteristics of the two bacteriophages are presented in Table 1.

Native preparations of host DNA banded in CsCl with a buoyant density of 1.696 g/cc. From the buoyant density measurement, the overall base composition is calculated to be 37 moles % guanine + cytosine (13, Fig. 1).

Both bacteriophage DNA in the native state banded as a unimodal peak in CsCl with a buoyant density of 1.691 g/cc (Fig. 2). When the DNA was denatured, two peaks of increased density, representing the complementary strands of the DNA, were obtained; their buoyant density was 1.705 g/cc and 1.710 g/cc. From the data, the overall base composition of the DNA from the two phases is identical, since the buoyant density measurements and the behavior of the denatured DNA were identical (Fig. 2). The ultraviolet absorbance-temperature profiles of native and denatured bacteriophage DNA, NCMB 385, are shown in Fig. 3.

From the Tm C value (81.5 C), the overall DNA base composition of NCMB 385 was calculated to be 30% guanine + cytosine.

**Table 1. Characteristics of two marine bacteriophages**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>NCMB 384</th>
<th>NCMB 385</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stability (pH)</td>
<td>7.4</td>
<td>7.4</td>
</tr>
<tr>
<td>Stability* at 4 C for 4 days in:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Distilled water</td>
<td>75</td>
<td>0</td>
</tr>
<tr>
<td>NaCl (3.8%)</td>
<td>56</td>
<td>80</td>
</tr>
<tr>
<td>MgCl₂ (0.3%) + NaCl (3.8%)</td>
<td>80</td>
<td>100</td>
</tr>
<tr>
<td>K value with:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>384 antiserum</td>
<td>154</td>
<td>120</td>
</tr>
<tr>
<td>385 antiserum</td>
<td>136</td>
<td>191</td>
</tr>
<tr>
<td>Latent period (min)</td>
<td>150</td>
<td>180</td>
</tr>
<tr>
<td>Burst size</td>
<td>28</td>
<td>20</td>
</tr>
</tbody>
</table>

* Survival in per cent.
FIG. 1. Microdensitometer tracing from an ultraviolet photograph of NCMB 397 purified native DNA taken after centrifugation to equilibrium in CsCl. The band of buoyant density 1.750 g/cm$^3$ is the density standard.

FIG. 2. Banding patterns of purified native and denatured DNA extract of bacteriophages NCMB 384 and 385. Reference standard is deuterated Escherichia coli K-12 DNA (1.750 g/cm$^3$). (Buoyant density 1.691 g/cm$^3$ = 31.6 moles % guanine + cytosine).

**DISCUSSION**

The preliminary data presented in this paper describe chemical and physical properties of two bacteriophages isolated from the marine environment and of the purified and highly polymerized DNA obtained from the two bacteriophages, NCMB 384 and 385. Previous studies of bacteriophages isolated from the marine environment have been limited to isolation and culture, with little attention paid to environmental parameters such as effect of temperature and salts on growth and reproduction of host and bacteriophage strains.

Tests to determine host specificity demonstrated that the bacteriophages lysed only the original host, NCMB 397, a marine Cytophaga sp. The bacteriophages, although isolated on separate occasions by Spencer (16) from seawater of the North Sea taken several miles from shore, were found to be serologically related, with minor differences most likely deriving from the protein components.

The studies on DNA from these two bacteriophages show an overall composition identical according to the methods employed (13, Fig. 2). The host DNA composition has been calculated at 37 moles % guanine + cytosine. The bimodal distribution in CsCl density gradients of the denatured bacteriophage DNA is similar to that observed for other bacteriophage DNA of low guanine + cytosine content (9). The two peaks in the density gradient indicate that the DNA is double-stranded, and from this evidence it can be assumed that the strands,
although complementary, are not equivalent in purine and pyrimidine content (Fig. 2 and 3). It would be interesting to determine the involvement of each of the complementary strands in the synthesis of complementary RNA and proteins. The stability of the bacteriophages in distilled water was very low (<10% viability), with both Mg$^{2+}$ and Na$^+$ required for retention of at least partial viability. Table 1 presents a summary of several experiments designed to test the stability of the bacteriophages in various media. In increasing order of efficiency of conferring stability on the bacteriophages, sodium, magnesium, and the combination of sodium and magnesium provided greater stability than distilled water. In artificial seawater, however, no significant decrease in titer was noted. It would be expected that free bacteriophages, to survive in seawater, must either be adapted to or resistant to the effects of the ionic constitution of their environment.

Single-burst analysis of the infected host revealed a rather low burst size, 28 for NCMB 384 and 20 for NCMB 385. Data from experiments with the single-step growth curve showed a 150-min latent period for NCMB 384 and 180 min for NCMB 385, when tested at room temperature (25°C). No indication of lysogeny has been observed.

Sufficient data have not yet been accumulated for bacteriophages isolated from seawater, seashells, or other habitats offered by the marine environment to correlate their morphology or chemical and physical composition with the broader ecological niche, the sea. Further study of NCMB 384 and 385, as well as of other marine bacteriophages, should provide information which will permit an understanding of how these forms survive and reproduce in the sea.

ACKNOWLEDGMENTS

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LITERATURE CITED