Isolation and Characterization of a Generalized Transducing Phage for the Marine Luminous Bacterium *Vibrio fischeri* MJ-1

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(Received 29 October 1986; revised 28 January 1987)

A marine bacteriophage active against the marine luminous bacterium *Vibrio fischeri* MJ-1 was isolated from offshore waters in Ensenada, Baja California, Mexico, and was shown to be active in generalized transduction, transducing 14 of 17 different amino acid auxotrophs to prototrophy. For some of the amino acid auxotrophic markers, such as arginine and methionine, several different mutants could be transduced. The phage grew well at temperatures up to 27 °C, and produced high-titre lysates (10^{10} p.f.u. ml⁻¹ or higher). Single-step growth analysis showed a latent period of 23 min at 25 °C with a burst size of 100. Phage adsorption was maximum at NaCl concentrations characteristic of the marine environment. No evidence for lysogeny was found.

INTRODUCTION

Phages attacking marine bacteria have been known for many years (ZoBell, 1946), but only relatively recently have detailed studies of their host range, physiology, structure and genetics been reported. Methods for the isolation and quantification of phages active against several different groups of marine bacteria have been published (Hidaka, 1971; Moebus, 1980; Koga *et al.*, 1982). The phages have been characterized by their host ranges (Valentine & Chapman, 1966; Moebus & Nattkemper, 1981), their ionic requirements (Spencer, 1960; Chen *et al.*, 1966; Nakamura *et al.*, 1978) and their fine structure (Valentine *et al.*, 1966; Sklarow *et al.*, 1973; Torella & Morita, 1979). Only a few genetic studies have been reported (Baross *et al.*, 1973; Keynan *et al.*, 1974), the latter presenting evidence for a specialized transducing phage active in transducing tryptophan auxotrophs of the marine luminous bacterium *Vibrio harveyi* to prototrophy. This phage was subsequently used to prepare a fine-structure genetic map of the *V. harveyi* tryptophan operon (Crawford & Nelson, 1976; I. P. Crawford & C. Beiger, unpublished).

The luminous bacterium *Vibrio fischeri* strain MJ-1 was originally isolated from the light organ of the luminous fish *Monocentris japonicus* (Ruby & Nealson, 1976). Recently the genes for bioluminescence from this strain were cloned and expressed in *Escherichia coli* (Engbrecht *et al.*, 1983), indicating that the *lux* genes, which code for the components necessary for luminescence, are located on a single contiguous area of the bacterial chromosome. Further studies have shown that the *lux* regulon consists of two operons that code for a total of seven proteins (Engbrecht & Silverman, 1984).

Other than cloning and expression of cloned genes in *E. coli*, no genetic studies have been reported in *V. fischeri*. For this reason, we initiated a search for bacteriophages that might be useful for genetic mapping purposes in strain MJ-1. We report here the isolation and characterization of a generalized transducing phage, rp-1, which has been used to transduce several different auxotrophs to prototrophy.

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METHODS

Growth of bacteria and isolation of phage. Strain MJ-1 was isolated from the light organ of the luminous fish *Monocentris japonicus* and identified as *Vibrio fischeri* (Ruby & Nealson, 1976). Several other strains of this species were also tested for phage sensitivity: CG-1 and CG-6, isolated from the light organs of two different specimens of the luminous fish *Cleidopus gloriamaris*; Y-1, a strain that emits yellow light (Ruby & Nealson, 1977); the type strain of *V. fischeri* (ATCC 7744); and 22 seawater isolates of *V. fischeri* obtained from coastal waters near Israel (12 from the Mediterranean Sea, 2 from the Red Sea, 8 from the hypersaline Bardawil Lagoon). All cultures were grown and maintained on SWC medium, consisting of 5 g Difco Bacto-Peptone, 0.5 g Difco Yeast Extract and 3 ml glycerol in 1 litre of 75% (v/v) seawater (Nealson, 1978). For experiments in minimal media, an artificial-seawater based medium, BGM, was used as previously described (Nealson, 1978). This medium contains, per litre of distilled water: 15.5 g NaCl; 0.75 g KCl; 12.4 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 1.45 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$; 50 ml Tris buffer stock (1 M, pH 7.5); 0.028 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$; 0.075 g $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$; 1 g NH_4Cl ; 3 ml glycerol.

Stock cultures were maintained on agar media (1.5%, w/v, Difco Bacto-agar) at 20 °C. Phages were grown and titrated by standard dilution methods in SWC 0.6% (w/v) top agar. These were done with 0.2 ml of exponential-phase bacteria plus 0.1 ml of the appropriate serial dilution of phage added to 4 ml of soft agar. After gentle mixing, these were poured over fresh SWC agar plates and incubated at room temperature (about 25 °C). Plaques were easily visible after 10–12 h.

Phage rp-1 was isolated from a sample of highly polluted seawater collected in August 1983 in the port of Ensenada, Baja California, Mexico. Yeast extract and peptone were mixed with the sample (200 ml) to make up SWC medium, and 1.5 ml of exponential-phase MJ-1 cells was added. This enrichment was shaken at room temperature for several hours. A lysate obtained by addition of 0.1 vol. chloroform was tested against strain MJ-1 and shown to contain 10^9 p.f.u. ml⁻¹. A pure strain of the phage was obtained by repeated isolation of single plaques from soft agar.

Phage preparation. Small-volume phage stocks were prepared by confluent lysis of plates containing approximately 10^3 plaques. Phage were washed off the plates in 5 ml sterile seawater, mixed vigorously on a vortex mixer, and centrifuged (8000 g, 10 min) to remove bacterial cells and debris. The supernatants were then collected and stored over 0.1 vol. chloroform at 4 °C. Titres of about 2×10^{10} p.f.u. ml⁻¹ were obtained by this method. Alternatively, 10^6 phage were inoculated on a soft agar (0.4%, w/v) lawn of MJ-1. After overnight growth, the lawn was harvested with a spatula, mixed with 0.1 vol. chloroform, and centrifuged directly. High-titre large-volume phage stocks were prepared by inoculating an SWC liquid culture of MJ-1 containing approximately 1.5×10^8 cells ml⁻¹ with phage rp-1 at a final concentration of 10^4 p.f.u. ml⁻¹ and aerating until lysis occurred. Using this method, titres in excess of 3×10^{10} ml⁻¹ were obtained; the stocks were then concentrated by high-speed centrifugation (35000 g, 1 h).

One-step growth experiments were performed according to Adams (1959). One ml of a rapidly growing culture (5×10^7 cells ml⁻¹) of MJ-1 was mixed with 1×10^7 phage particles. After adsorption (5 min) this mixture (10 µl) was diluted in 200 ml SWC medium in a 500 ml Erlenmeyer flask, and gently shaken at 25 °C. At 2 min intervals, two samples (0.1 ml) were removed and assayed for p.f.u.

Auxotrophs. Auxotrophic mutants of MJ-1 were isolated after treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) (Sigma). Overnight cultures were washed twice in sterile seawater, resuspended in sea water at a concentration of 10^9 cells ml⁻¹ and allowed to stand (room temperature, 1 h). A small crystal of MNNG was then added, and at intervals (10 min), 30 SWC plates were spread with 0.1 ml of a 10^4 dilution of the mutagenized cells so that at 1% survival the plates yielded 100 colonies per plate. Using this approach, mutagenesis of the surviving colonies was excellent, and several auxotrophs could be picked from each plate by replica plating to SWC and BGM plates. The auxotrophs were identified by replica plating to BGM plates containing mixtures of amino acids.

Transduction. Exponentially growing cultures of auxotrophic mutants (about 5×10^8 cells ml⁻¹) were infected with phages that had been irradiated with UV light to 1% survival. An m.o.i. of 3 (assayed before irradiation) was used for maximum transduction. Phages were adsorbed (8 min, room temperature) and 5×10^7 bacteria were plated directly on BGM. Controls containing phage and recipient bacteria individually were also plated. Colonies were counted after incubation for 3–4 d at room temperature.

Electron microscopy. A drop of seawater containing rp-1 ($> 10^{12}$ p.f.u. ml⁻¹) was placed on a carbon-coated grid, dried, washed twice with distilled water, negatively stained with 2% (w/v) uranyl acetate, and then used directly for electron microscopy as previously described (Keynan *et al.*, 1974).

DNA extraction. DNA was extracted and precipitated from rp-1 according to Maniatis *et al.* (1982), with the exception that the chloroform extraction step was omitted. After storage at -20 °C, samples were centrifuged (9000 g, 40 min), and the pellets lyophilized for several minutes until visibly dry. These were then resuspended in TE buffer (10 mM-Tris/HCl, pH 5.0, 1 mM-EDTA), incubated at 65 °C for 10 min and mixed on a vortex mixer to ensure complete recovery. These stocks were stored at -20 °C.

Table 1. *Characteristics of bacteriophage rp-1*

Shape and size*	Head: appears hexagonal; probably icosohedral; 83 nm diameter Tail: long, straight, contractile; length 83 nm; width 16 nm
$s_{20,w}$ of phage†	852
Density of phage†	1.525 g ml ⁻¹
DNA‡	48 MDa
Adsorption time	>95% in 5 min
Burst time	23 min at 25 °C
Burst size	100 p.f.u. per bacterium infected
Host range (strains sensitive/ strains tested)‡	<i>E. coli</i> , 0/4; <i>V. harveyi</i> , 0/46; <i>V. cholerae</i> , 0/1; <i>V. splendida</i> , 0/2; <i>V. fischeri</i> , 21/27; <i>Xenorhabdus luminescens</i> , 0/3; <i>Photobacterium phosphoreum</i> , 0/9; <i>Photobacterium leiognathi</i> , 0/19; <i>Alteromonas hanedai</i> , 0/1

* Size estimates are means of six measurements of intact phage particles of known magnification. For intact phage, sizes were reproducible to within 5%.

† Phage density and $s_{20,w}$ determinations were done by preparative density-gradient centrifugation of whole phage, and analytical density-gradient centrifugation of DNA, respectively, as described by Keynan *et al.* (1974). DNA size was estimated by agarose gel electrophoresis with known standards (Maniatis *et al.*, 1982).

‡ Droplets of 2 µl containing a total of 2×10^6 p.f.u. of phage rp-1 were spotted onto freshly prepared lawns of bacteria to be tested, in fresh, soft-agar overlays. Lysis was visually checked after 1 d incubation at room temperature.

RESULTS AND DISCUSSION

The isolation of rp-1 was unusually difficult in comparison to many of the phage isolations we have previously done. Many attempts were made on a variety of different samples off Southern California, where *V. fischeri* is known to occur in abundance (Ruby & Nealson, 1977). The reasons for this are not known. Similar attempts off the coast of Israel yielded *V. fischeri* phages relatively easily.

Characteristics of phage rp-1

The host range and other general characteristics of rp-1 are shown in Table 1. Many strains of marine bacteria were tested for sensitivity to phage rp-1, including *Vibrio harveyi*, *Photobacterium phosphoreum*, *Photobacterium leiognathi*, *Alteromonas hanedai*, and several non-luminous species. Only strains of *V. fischeri* were sensitive, and of 27 strains tested, 21 were sensitive to rp-1. The non-sensitive strains included CG-1, CG-6, Y-1 and ATCC 7744 as well as one strain each from the Mediterranean Sea and the Red Sea. All other *V. fischeri* isolates tested were sensitive to lysis by phage rp-1.

One criterion that has been used to characterize marine phages is that of specific ionic requirements (Spencer, 1960), and although rp-1 was isolated from the marine environment and is specific for a marine bacterial host, it differs markedly from other marine phages with respect to ionic stability. Even in distilled water, where other marine phages are lysed within minutes (Keynan *et al.*, 1974) rp-1 was stable for 24 h. Although inactivation did not occur with changes in ionic strength, phage adsorption was affected (Fig. 1). Adsorption was maximum at 3% (w/v) NaCl, near the total salinity of seawater, and decreased rapidly at either higher or lower NaCl concentrations.

Although rp-1 was stable at temperatures of 35 °C and below, it was rapidly inactivated by temperatures of 45 °C or greater. Temperature also affected plaque morphology; at 23 °C, plaques were clear (or slightly hazy) with well-defined edges. At 30 °C, plaques formed, but they were very opaque (hazy) with poorly defined edges. In some cases the centres of these plaques had clear zones.

Fig. 2 shows the effect of phage infection on growth and luminescence of strain MJ-1; there was no significant effect on light emission until cell lysis occurred. In no case was it possible to show lysogeny of MJ-1 by rp-1. Supernatants of phage-resistant cultures were screened by the soft-agar overlay method, and they carried no phage.

Fig. 1. An exponential-phase culture of MJ-1 (1.5×10^8 cells ml^{-1}) was harvested by centrifugation and resuspended at the same density in distilled water containing NaCl at various concentrations. Phage rp-1 was added to these suspensions at an m.o.i. of 0.05. At the indicated times samples were removed, shaken with chloroform and subsequently titrated to determine the phages not removed by adsorption to the bacteria. NaCl concentrations (% w/v): ∇ , 8; $\triangle$, 6; $\square$, 5; $\circ$, 4; $\blacktriangledown$, 3; $\blacktriangle$, 2; $\blacksquare$, 1; $\circ$, 0.5.

Fig. 2. An exponential-phase culture of MJ-1 (1.5×10^8 cells ml^{-1}) at 23°C was divided into two portions, and phage rp-1 was added to one ($\square$, $\blacksquare$) at an m.o.i. of 3. At the indicated times, culture density ($\circ$, $\square$) and bioluminescence ($\bullet$, $\blacksquare$) were monitored. Luminescence measurements were done according to Ruby & Nealson (1977).

Transduction experiments

With the advent of molecular genetic approaches, the search for transducing phages and other more classical genetic alternatives might well be questioned. However, in systems such as the marine bacteria, alternative genetic systems, such as transducing phages, are of potentially great value. Part of this value lies in the fact that there are aspects of the physiology and regulation of

Table 2. Transduction of MJ-1 auxotrophs by phage *rp-1* grown on wild-type MJ-1

Mutant designation*	Revertants†	Transductants†	Mutant designation*	Revertants†	Transductants†
<i>arg-2</i>	0.0	40	<i>leu-3</i>	0.0	12
<i>arg-4</i>	10.0	37	<i>lys-1</i>	0.7	56
<i>asn-1</i>	2.7	35	<i>met-1</i>	10.0	49
<i>cys-1</i>	0.0	30	<i>met-2</i>	11.0	40
<i>cys-3</i>	0.3	41	<i>met-5</i>	42.0	96
<i>cys-4</i>	0.7	43	<i>thi-1</i>	1.3	27
<i>cys-6</i>	1.0	36	<i>thr-1</i>	0.0	23
<i>gly-3</i>	0.3	13	<i>trp-2</i>	4.0	29
<i>his-2</i>	4.3	45	<i>tyr-1</i>	4.0	40
<i>ile-2</i>	0.0	11	<i>tyr-2</i>	0.3	106
<i>ilv-3</i>	0.3	29			

* No transductants were obtained for the following mutants: *arg-1*, -3, -5, -6, -7; *cys-2*, -5; *gln-1*, -2; *leu-1*, -2, -4; *met-3*, -4; *orn-1*; *pro-1*, -2.

† Numbers are the mean of three experiments, and represent the number of revertants or transductants per 5×10^7 infected recipients plated.

Table 3. Transduction of MJ-1 mutants by *rp-1* grown on auxotrophs

Mutant transduced	Revertants*	Transductants* with phage grown on:			
		Wild-type	<i>arg-2</i>	<i>cys-6</i>	<i>tyr-2</i>
<i>arg-2</i>	0.0	18	0.0	30	26
<i>arg-5</i>	0.0	0.3	0.0	0.0	0.3
<i>asn-1</i>	2.3	54	55	15	103
<i>cys-4</i>	2.7	48	28	0.0	72
<i>cys-6</i>	3.7	39	18	3.7	53
<i>lys-1</i>	0.0	74	57	54	17
<i>trp-2</i>	2.7	29	28	20	59
<i>tyr-2</i>	0.0	103	75	106	0.3

* Numbers are the mean of three experiments, and represent the number of revertants or transductants per 5×10^7 infected recipients plated.

the luminous system that differ between *E. coli* and naturally luminous bacteria, so that a complete understanding of the luminous system will depend on studies done in strains from which the genes were obtained. For example, in MJ-1, luminescence is strongly repressed by iron, while no iron repression of luminescence is seen with the cloned genes in *E. coli* (Haygood & Nealson, 1985).

Table 2 is a compilation of transduction experiments done using phage *rp-1* prepared on wild-type MJ-1 cells. Of 38 different auxotrophic isolates, 21 were transduced to prototrophy; these included 14 of 17 different markers, with several positives for some markers. Transduction efficiencies varied by about a factor of 10 between mutants, with the best efficiency shown by *tyr-2*. This mutant also had a low reversion rate and was thus the mutant of choice for further experiments. All transductants tested remained sensitive to phage *rp-1* and none released any detectable phage into the medium.

Several other experiments were done in order to test the hypothesis that the increased reversal to prototrophy was indeed due to transduction. Phage lysates used for transduction were tested for the presence of live bacteria by streaking onto SWC plates. None were found, ruling out the possibility of contamination or conjugation. Treatment of *rp-1* lysates with pancreatic DNAase under conditions which led to maximum hydrolysis of calf thymus DNA had no effect on the ability of these lysates to transduce, ruling out the possibility of transformation. Finally, an *rp-1*-resistant mutant of *tyr-2*, which showed no adsorption of phage *rp-1*, was not reverted to prototrophy by exposure to *rp-1*.

Table 3 shows the results of transduction experiments using phage preparations grown on

several different auxotrophic strains. In no case was it possible to transduce an auxotrophic mutant with phage grown on that strain. Mutant *cys-4* was transduced by phage grown on three other auxotrophs, but not by phage grown on either *cys-4* or *cys-6* auxotrophs, suggesting that these mutations were identical or very closely linked.

No wide-host-range plasmids have yet been successfully transformed into and maintained in strain MJ-1. The availability of *rp-1*, which clearly replicates well in MJ-1, opens the possibility of engineering its DNA for use as a genetic vector in this strain. Until this report, no generalized transducing phages had been reported for *V. fischeri*. The success obtained in transducing auxotrophs with phages grown on a variety of different mutants suggests that the phage could be used for genetic studies including fine-structure mapping. Such studies would nicely complement other methods of genetic analysis.

This work was done while R. L. was on sabbatical leave from Tel Aviv University. It was supported in part from grants to K. H. N. from the Office of Naval Research (N00014-80-C0066) and from the Milwaukee Foundation. This publication is no. 295 from the Center for Great Lakes Studies.

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