CURRENT MICROBIOLOGY Vol. 17 (1988), pp. 49–53

Current Microbiology

Isolation and Characterization of Phage F9-11 from a Lysogenic *Deleya halophila* Strain

Concepción Calvo, Ana García de la Paz, Victoria Bejar, Emilia Quesada, and Alberto Ramos-Cormenzana

Department of Microbiology, Faculty of Pharmacy, University of Granada, Granada, Spain

Abstract. Thirty-eight strains of *Deleya halophila* species were examined for production of phage after mitomycin C induction. Thirty-two of them were able to inhibit growth of some other strains. Phage F9-11, isolated from *D. halophila* strain F9-11, showed an isometric head and a noncontractile tail. The effects of salt concentrations variation on the stability and replication of this phage were established. Its replication was possible at a wide range of marine salt concentrations, from 2.5% to 15% (wt/vol). Stability seems to be influenced by osmolarity of medium rather than by NaCl level. The euryhaline character showed by F9-11 phage is evoked as an important factor for the survival of this phage in its environment.

Deleya halophila is a new species of moderately halophilic bacteria isolated and described by Quesada et al. [5] from a hypersaline soil located near Alicante (Spain). This microorganism is able to grow in a wide range of salt concentrations (between 2.5% and 25%, wt/vol total salts) and presents its optimal growth at 7.5% (wt/vol) when grown at 32° and 42°C incubation temperatures. This bacterium was found to have an absolute requirement for the Na^+ cation, that it could not be replaced by other cations or by nonionizable solutes [6].

In contrast to the extensive studies performed on extremely halophilic microorganisms, numerous questions remain on moderately halophilic bacterial ecology, physiology, and taxonomy.

This paper reports the first isolation of a bacteriophage from a lysogenic *Deleya halophila* strain and how salinity affects the stability and replication of this phage.

Materials and Methods

Bacterial strains. Thirty-eight strains of the moderate halophile *Deleya halophila* isolated from hypersaline soils by Quesada et al. [5] were examined for the presence of a prophage.

Research of lysogeny. Attempts to demonstrate lysogeny were performed by techniques described by Adams [1]. Exponentially growing cultures in MH liquid medium [4] containing 7.5% (wt/vol) marine salts were treated with 2.5 g/ml of mitomycin C (Sigma) and incubated at 32°C for 5 h, followed by centrifugation at 3000 rpm for 30 min. The supernatants were filtered through a 0.22 μm membrane filter (Millipore Corp.). Filtrates were tested for activity by the double-agar layer method [1] on each *D. halophila* strain included in this work.

Isolation and propagation of phage F9-11. Phage F9-11 was isolated from the lysogenic *D. halophila* strain F9-11, and it was purified three times by repeated single plaque propagation on *D. halophila* strain G-2.

Adsorption of phage F9-11. The rate of phage adsorption was studied by assaying for unadsorbed phage as described by Adams [1]. Adsorption was determined at six different salt concentrations (%, wt/vol): 0, 2.5, 5, 7.5, 10, and 15. Host bacteria (*D. halophila* strain G-2) was grown in MH liquid medium at 7.5% (wt/vol) and then centrifuged at 5000 rpm for 30 min and washed with MH liquid medium at the appropriate salt concentration. Phage was added to a bacterial suspension at a multiplicity of infection of 1, and readsorption of the phage was prevented by dilution. Adsorption period was 30 min, and incubation temperature was 32°C.

One-step growth. The procedure for one-step growth experiments was similar to that of Adams [1]. It was also determined (as adsorption rate) at seven different salt concentrations (%, wt/vol): 0, 2.5, 5, 7.5, 10, 15, and 20. Host bacteria (*D. halophila* strain G-2) was grown to a density of 10^8 cells/ml in MH liquid medium at 7.5% (wt/vol) and then centrifuged at 5000 rpm for 30 min and washed with MH liquid medium of the desired salt concentration.

Phages were added at a multiplicity of infection of 1 and incubated at 32°C for 15 min; 0.1 ml aliquot was then removed from the flask and added to 10 ml of fresh medium to stop the adsorption and filtered through a 0.22-μm membrane filter (Milli-

Address reprint requests to: Dr. C. Calvo, Department of Microbiology, Faculty of Pharmacy, C/ Rector López-Argüeta s/n, 18001 Granada, Spain.

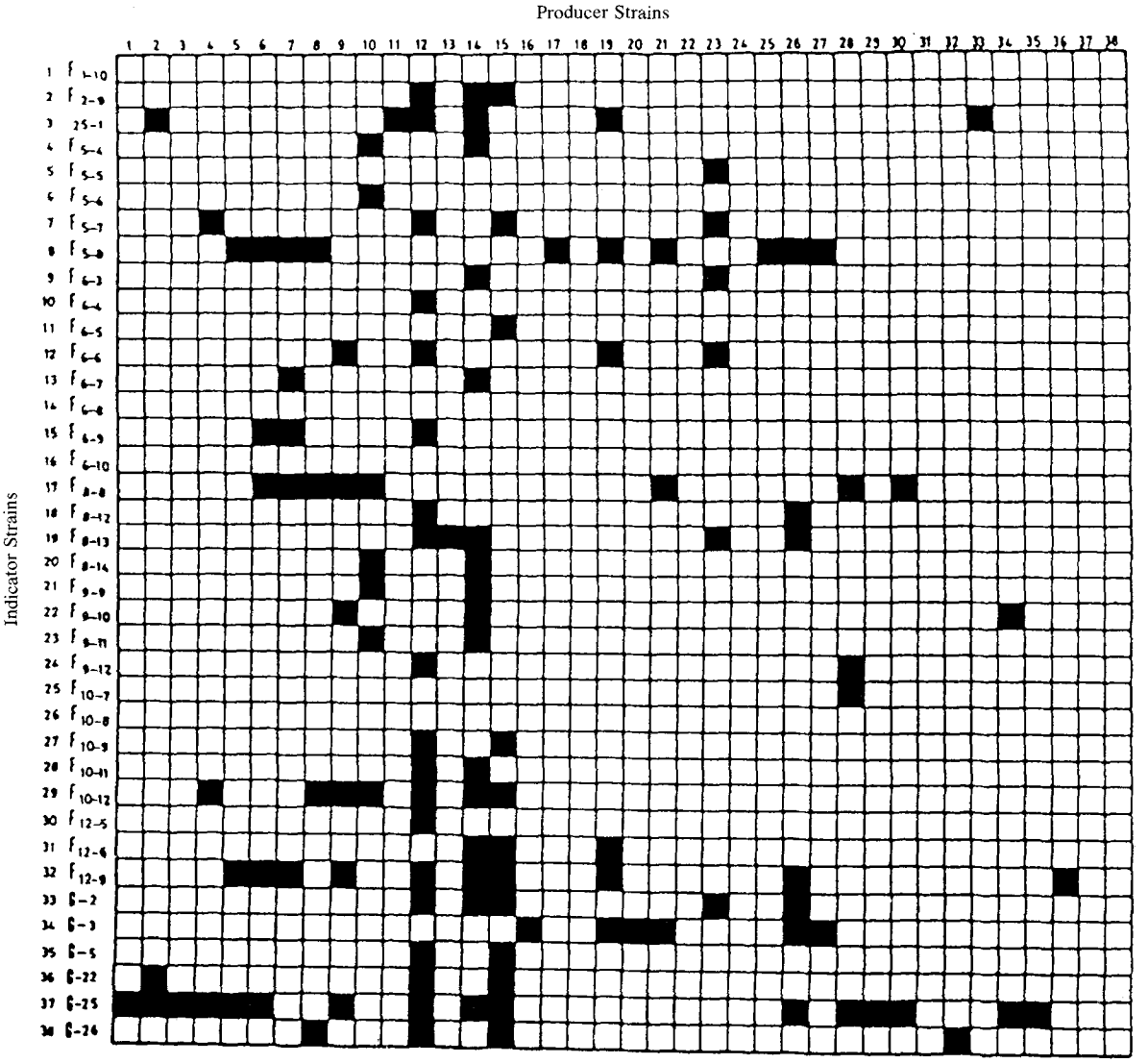

Fig. 1. Reciprocal test of *Deleya halophila* strains for growth-inhibiting activity: ■, inhibition; □, no inhibition. The numbers given to producer strains correspond to those of indicator strains.

pore Corp.) in order to eliminate unadsorbed phage. Membrane filter was then resuspended in the same volume (10 ml) of fresh medium and dilution 1/100 and 1/10,000 were made to get the first and second growth tubes. Samples of 0.1 ml were subsequently assayed for total plaque-forming units (PFU).

Survival experiments. Survival of phage F9-11 was studied in MH liquid medium and in Subow solution [7] at different salt concentrations (%, wt/vol): 2.5, 5, 7.5, 10, and 15. Phage dilutions were made in 15 ml of the appropriate medium and stored at 4°C. Periodically the titer was determined by plaque assay.

Electron microscopy. Phages were sedimented at 30,000 rpm for 90 min in a Beckman L8-70 ultracentrifuge with a rotor type 70 Ti and resuspended in 0.5 ml distilled water. The phage suspension was applied to carbon-coated grids, fixed, and negatively stained with 1% (wt/vol) uranyl acetate. The grids were examined in an EM 10C/CR electron microscope.

Results

Lysogeny. Thirty-two of the 38 strains studied were able to inhibit growth of some other strains. However, 12 of them showed a bacteriocin-like activity, since lytic plaques were never observed when the filtrates were titrated. The most active strains were *D. halophila* F6-6 (it inhibited 19 strains) and *D. halophila* F6-8 (it inhibited 17 strains). In contrast, *D. halophila* F8-12, F9-10, F9-12, F12-6, G-25, and G-26 strains showed no activity (Fig. 1).

Phage F9-11 isolated from *D. halophila* strain F9-11 was selected in order to do its partial characterization. This phage formed clear plaques of 0.5–1 mm in diameter without halo in double agar layer at 5% (wt/vol) salt concentration, but it was unable to form plaques at salt concentrations higher than 7.5% (wt/vol). The optimal temperature was 32°C.

Rate of adsorption. Adsorption rate of phage F9-11 was dependent on salt concentrations (Fig. 2). Optimum adsorption (98%) was reached at 5% (wt/vol) and decreased to 35% adsorbed when salt concentration was increased from 5% to 15% (wt/vol). At low salt concentration (2.5%, wt/vol) phage F9-11 adsorption was low (40% adsorbed phage).

One-step growth. The growth curves for phage F9-11 carried out in 2.5%, 5%, 7.5%, 10%, and 15% (wt/vol) salt concentrations are shown in Figure 3. Replication varied with changes in salt concentrations, and influence on replication was reflected in changes in both latent period and burst size. Negative results were obtained when single-step growth experiments were performed at 0% and 20% (wt/vol).

Survival experiments. The results of studies on inactivation of phage F9-11 in both solutions and MH liquid medium at different salt concentrations have shown that stability at 30% and 15% (wt/vol) salt concentrations was higher in Subow solution than in MH liquid medium. In contrast, at low salt concentrations titers decreased faster in Subow solutions than in MH liquid medium (Fig. 4).

Morphology. Electron microscopic examination of the negatively stained phage F9-11 revealed that it was composed of an isometric head and a noncontractile tail. According to this morphology, phage F9-11 could be included in the Family *Styloviridae* (Fig. 5).

Discussion

Moderately halophilic bacteria are defined as those bacteria which grow optimally at NaCl concentrations between 3% and 15% (wt/vol). Whereas bacteriophages active on extremely halophilic bacteria [8, 9] and on marine bacteria [2, 10] have been reported, the existence of phages active on moderate halophiles has never been described.

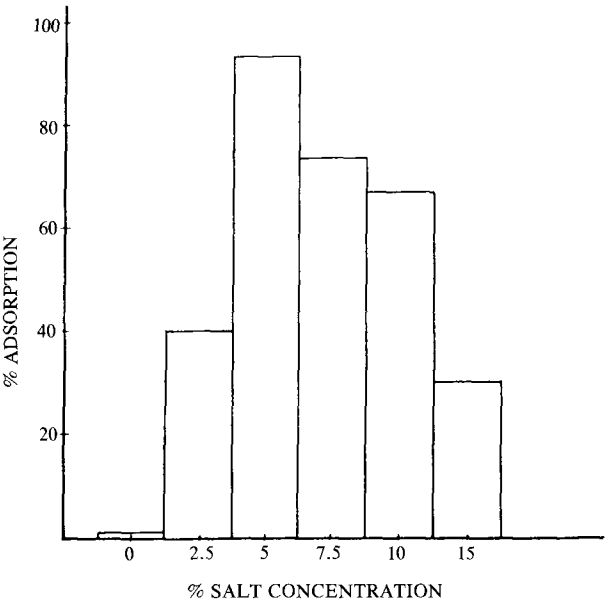

Fig. 2. Rate of adsorption of bacteriophage F9-11 to *Deleya halophila* strain G-2 at various salt concentrations.

52.5% (28/38) of filtrates from *D. halophila* strains were able to produce isolated lytic plaques on strains belonging to the same species. To our knowledge *D. halophila* is the first moderately halophilic species described as producer of temperate phages, perhaps owing to the difficulty of lytic plaque observation.

The criteria that have proved most useful in distinguishing halophilic from nonhalophilic phages were the physiological characteristics of phage replication, especially with respect to factors important in their environment.

Influence of salt concentrations on replication and survival of phage F9-11 isolated from the lysogenic *D. halophila* strain F9-11 was studied. Its replication was possible at a wide range of marine salt concentrations, from 2.5% to 15% (wt/vol). Rate adsorption was maximal at 5% (wt/vol), a lower value than the host cell optimum (7.5%, wt/vol), and phage production was maximal at 10% (wt/vol). On the other hand, the long latent period found at 10% (wt/vol) may be the reason for the lower adsorption shown in Figure 1 compared with adsorption rates at other salt concentrations.

Deleya halophila shows a strong euryhaline character, with a range of salinity wider than that determined for moderately halophilic microorganisms [3, 5]. Our data on the effect of salt concentration on the multiplication of phage F9-11 indicate

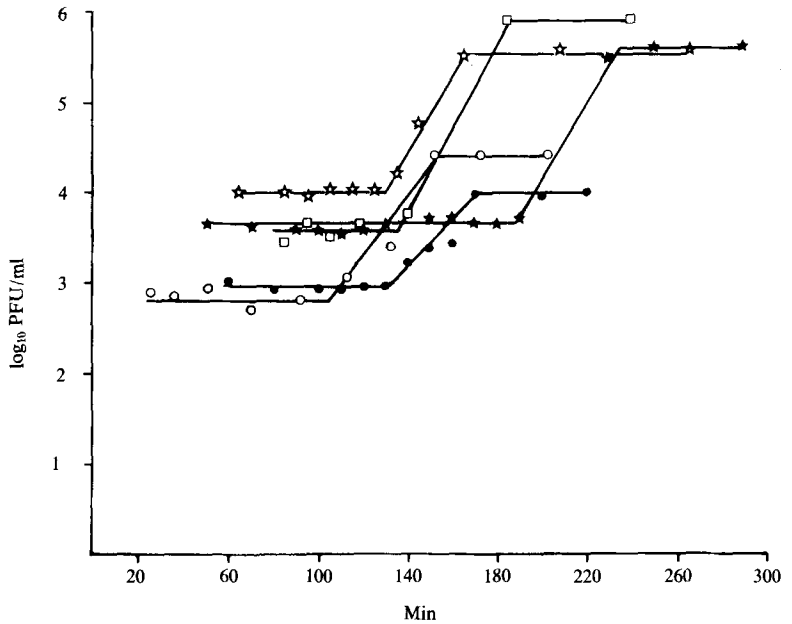

Fig. 3. One-step growth of phage F9-11 at different salt concentrations: ☆—☆, 2.5% (wt/vol); ○—○, 5% (wt/vol); ●—●, 7.5% (wt/vol); □—□, 10% (wt/vol); ★—★, 15% (wt/vol).

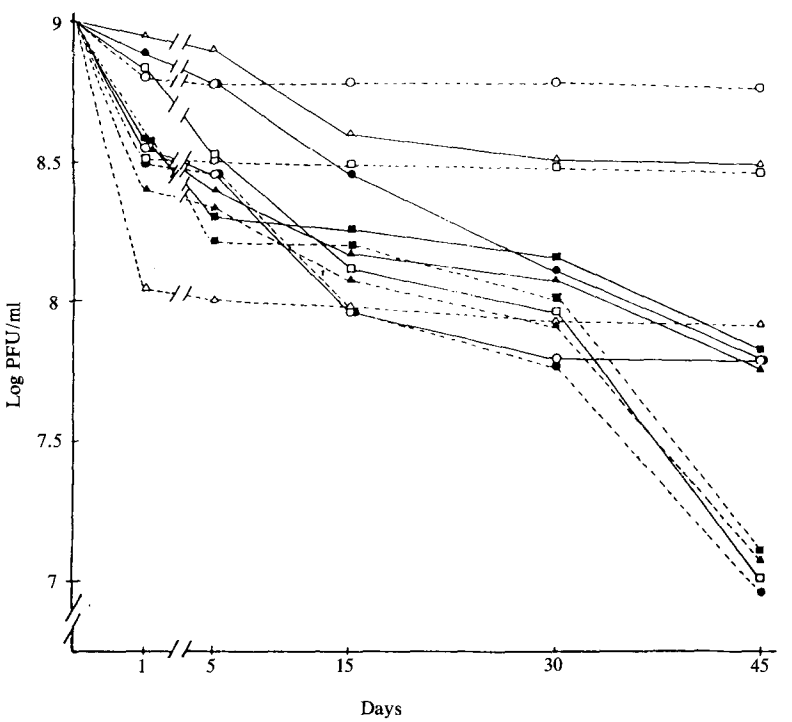

Fig. 4. Survival of phage F9-11 diluted in both MH liquid medium and Subow solution, at various salt concentrations. ○---○, 30% Subow; ○—○, 30% MH; □---□, 15% Subow; □—□, 15% MH; △---△, 7.5% Subow; △—△, 7.5% MH; ●---●, 5% Subow; ●—●, 5% MH; ■---■, 2.5% Subow; ■—■, 2.5% MH; ▲---▲, 0% Subow; ▲—▲, 0% MH.

the same euryhaline character for this bacteriophage.

Survival experiments seem to demonstrate two important features; first, that stability of phage F9-11 is influenced by osmolarity of medium rather than by NaCl level, and second, that free phage particles stay viable during a long period of time (45 days) at a wide range of salt concentrations (from 0% to 30%, wt/vol).

One possible explanation for the euryhaline character of the phage F9-11 might be the heterogeneity of soil habitat, from which *D. halophila* has been isolated, where the salinity can change markedly in space and time [3]. As a consequence, eury-

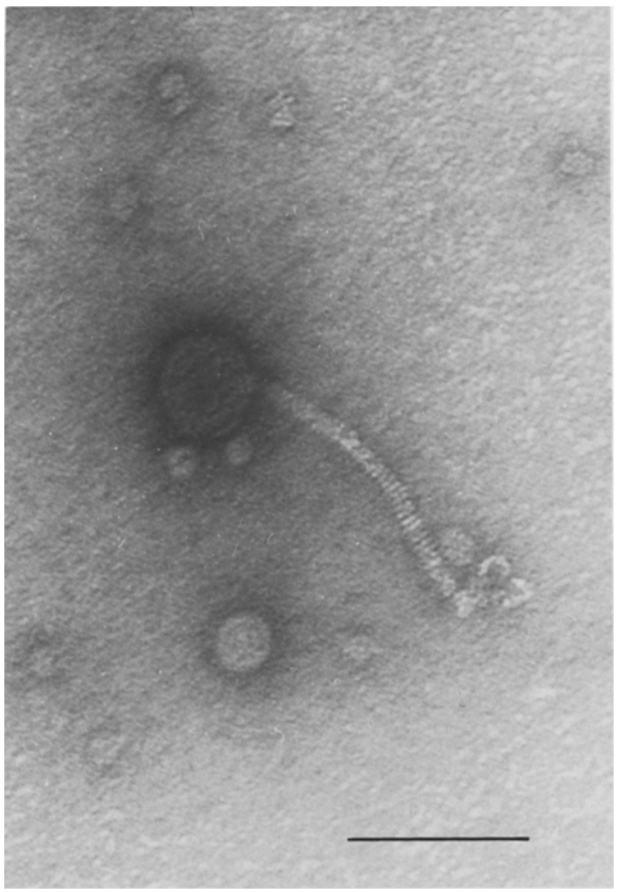

Fig. 5. Phage F9-11 negatively stained with 1% (wt/vol) uranyl acetate. Bar = 0.1 μm.

haline bacteriophages, like euryhaline bacteria, might be consistently favored.

Literature Cited

1. Adams MH (1959) Bacteriophages. New York: Wiley-Interscience
2. Baross JA, Liston J, Morita RY (1978) Incidence of *Vibrio parahaemolyticus* and other *Vibrio* bacteriophages in marine samples. Appl Environ Microbiol 36:492–499
3. Ferrer MR, del Moral A, Quesada E, Ramos-Cormenzana A (1987) Growth rate and some physiological features of *Deleya halophila* CCM 3662^T at different salt concentrations. Ann Inst Pasteur/Microbiol 138:49–57
4. Quesada E, Ventosa A, Rodriguez-Valera F, Megias L, Ramos-Cormenzana A (1983) Numerical taxonomy of moderately halophilic Gram-negative bacteria from hypersaline soils. J Gen Microbiol 129:2649–2657
5. Quesada E, Ventosa A, Ruiz-Berraquero F, Ramos-Cormenzana A (1984) *Deleya halophila*, a new species of moderately halophilic bacteria. Int J Syst Bacteriol 34:287–292
6. Quesada E, Bejar V, Valderrama MJ, Ramos-Cormenzana A (1987) Growth characteristics and salt requirements of *Deleya halophila* in a synthetic defined medium. Curr Microbiol, in press
7. Subow NN (1931) Oceanographical tables. Moscow: URSS Oceanographic Institute hydrometeorological Commission.
8. Torsvik T, Dundas ID (1980) Persisting phage infection in *Halobacterium salinarium* str 1. J Gen Virol 47:29–36
9. Wais AC, Kon M, Mac Donald RE, Stollar BD (1975) Salt-dependent bacteriophages infecting *Halobacterium cutirrubrum* and *H. halobius*. Nature 256:314–315
10. Zachary A (1976) Physiology and ecology of bacteriophages of the marine bacterium *Beneckea natriegens*: salinity. Appl Environ Microbiol 31:415–422