Bacteriophages of psychrophilic pseudomonads. II. Host range of phage active against *Pseudomonas putrefaciens*

A. L. DELISLE AND R. E. LEVIN

Department of Food Science and Technology, University of Massachusetts, Amherst, Mass. 01002 *U.S.A.*

DELISLE, A. L. and LEVIN, R. E. 1969. Bacteriophages of psychrophilic pseudomonads. II. Host range of phage active against *Pseudomonas putrefaciens*. Antonie van Leeuwenhoek **35**: 318–324.

Forty phages active against 4 terrestrial and against 10 of 24 marine strains of *P. putrefaciens* were isolated from raw municipal sewage, fish-pier water, and refrigerated haddoch fillets. With one exception, phages active against marine strains were obtained only from pier water and fish fillets. Only two strains were attacked solely by their homologous phage while five of the marine strains were not attacked by any phage. Lytic reactions revealed no obvious differences between marine and terrestrial strains.

Detectable bacteriocins were not produced by any of the strains and only one strain was found to be lysogenic. Most of the phages were capable of forming plaques at 2 C, regardless of the temperature at which they were isolated.

INTRODUCTION

P. putrefaciens (*Achromobacter putrefaciens*) was originally isolated by Derby and Hammer (1931) from tainted butter. Long and Hammer (1941) subsequently isolated this species from moist soil, stream and lake water, roadside water, and from dairy plants. They studied the major biochemical characteristics of this species, which were used to describe the species in Bergey's Manual (Breed, Murray and Smith, 1957).

Castell, Richards and Wilmot (1949) were the first to report that *P. putrefaciens* was involved in the spoilage of marine fish, in this case, cod fillets. Studies in our laboratory have indicated that *P. putrefaciens* is also a potent spoiler of haddock and constitutes a significant proportion of the spoilage flora usually found on refrigerated haddock fillets (Chai et al., 1968).

Since information on marine strains is practically non-existent, a detailed study of this species was initiated. As part of this study bacteriophage relation-

[1] The present work represents part of a dissertation submitted by the senior author in partial fulfillment of the requirements for the Ph.D. degree at the University of Massachusetts.

ships were investigated, with the objective of comparing marine strains isolated from fish with known terrestrial strains.

MATERIALS AND METHODS

Bacterial methods. Nutrient salts broth media (NBS) used in this study are described in the previous paper (Delisle and Levin, 1969). Identification of *P. putrefaciens* strains was based on the description given in Bergey's Manual (Breed et al., 1957), and additional characteristics as described by Levin (1968). The ability to produce H_2S (which was not mentioned by earlier workers), while highly characteristic, is not completely stable and is frequently lost by some strains. Anaerobic growth in the medium of Hugh and Leifson (1953) also distinguishes this species from the usual aerobic, gram-negative, polarly flagellated rods commonly found on fish fillets.

The strains used in this study and their sources are as follows: S2 (terrestrial; from W. E. Sandine); 8071, 8072, 8073 (terrestrial; from ATCC); Syl 1, Syl 2 (isolated from haddock fillets in this laboratory, 1965); Pl through P17, P19, P19x, P20, and P21 (isolated from haddock fillets in this laboratory, 1967; Chai et al., 1968).

Fluorescent pigment production has been encountered with only one strain, ATCC culture 8071, and then only on media supplemented with gelatin or RNA, but not DNA. *P. putrefaciens* 8073 does not grow in the temperature range of 0-4 C and strain 8072 grows very slowly at 2 C on agar media. With the exception of these 2 strains, however, all *P. putrefaciens* strains are potent spoilers of refrigerated fresh fish fillets (Delisle and Levin, 1969). The "P" series of strains were selected on the basis of differences in colony appearance and degree of black coloration on Peptone-Iron agar plates (Difco) made from haddock fillet homogenates. Among these strains P19 and P20 produced no H_2S but did produce typical salmon colored colonies, and strain 8072 subsequently lost the ability to produce H_2S.

Phage methods. Procedures used for isolating and purifying phage have been described (Delisle and Levin, 1969). Sources used for isolating phage were raw municipal sewage, seawater from alongside the Boston Fish Pier (pier water) and spoiled haddock fillets stored at 4 C for 3–4 weeks. Three attempts were made to isolate phage from each source for each strain. Duplicate enrichments were made at 2 C and 20 C, using both pier water and sewage; enrichments from fillets were incubated only at 2 C.

Five methods were used to detect temperate phage. In the first method supernatants of NSB broth cultures were spotted onto freshly seeded top agar plates of each strain. The second method consisted of heating early log-phase

NSB cultures at 45 C or 55 C for 5–15 minutes and then testing supernatants for the presence of phage after additional incubation at 2 C or 20 C. In the third method H_2O_2 was added to log phase cultures to a final concentration of 0.01 to 0.1% (v/v) and the supernatants were again tested after several hours incubation at 20 C. Ultraviolet radiation (500 μW/cm^2) of log phase cultures was used for the fourth method. The fifth method involved addition of mitomycin C (Nutritional Biochemicals Corp.) to mid-log phase NSB cultures (5×10^7 cells/ml) at a final concentration of 1 μg/ml. After 8 hours incubation at 20 C or 40 hours at 2 C, supernatants were tested on all the other *P. putrefaciens* strains for the presence of phage. In each method $CHCl_3$ was added to lyse the cells and release any internal, mature phage which might otherwise have remained undetected.

The phage typing procedures used were the standard routine test dilution (RTD) method as described by Blair and Williams (1964). After determining the dilution required to give just confluent lysis on its propagating strain (RTD dilution) the host range was determined by spotting 0.05 ml of this dilution, by means of a standard inoculating loop, onto freshly seeded top agar plates of each test strain. To determine effects of host pre-growth temperature and plate incubation temperature, a duplicate set of plates seeded with inocula grown at 2 C was prepared. After the spots had dried (30–60 min) one set of plates was incubated at 2 C and the other at 20 C. This procedure was also followed using inocula grown at 20 C. Each typing experiment was repeated two or more times. Negative reactions were confirmed by spotting undiluted lysates directly onto seeded plates. No plaques or inhibition of growth was seen when using those lysates which did not form plaques on a given test host at RTD.

RESULTS

Isolation of phage. Forty phages were isolated for 14 of the 27 *P. putrefaciens* strains. In several cases phages were obtained only at one temperature, even though phage enrichment cultures were incubated at both 2 and 20 C.

Phage could not be obtained from sewage for any of the 23 marine strains except strain Syl 2. Neither was it possible to obtain phage for the terrestrial strains from fillets.

Host range of phage. Although phages were not isolated for 13 of the strains only 5 were resistant to all 40 phages (Table 1). Phage No. 15 and No. 16 also attacked 2 strains of *P. fluorescens* and one strain of *P. putida* (Delisle and Levin, 1969; not shown in Table 1). These two phages are thus quite different from all the others isolated. It is also possible that the original host of these 2 phages, *P. putrefaciens* 8072, is significantly unique, even though its lytic pattern is

similar to those of strains S2 and P2. Two strains, P6 and P11, were attacked only by their homologous phage. Strain P2 is the most sensitive indicator strain for the phages isolated, being attacked by 23 of the 40 phages and appears to be closely related to strain S2.

Some similarities between the lytic patterns of marine and terrestrial strains are evident and no obvious differences were revealed. It was not possible to differentiate terrestrial strains from marine strains except in cases where lytic patterns exactly matched. In view of the discrete differences between all strains (each has an unique lytic pattern) however, specific strains can be precisely identified.

Detection of lysogeny. The failure to isolate phage which can attack the five resistant strains (P4, P5, P8, P15, and P16) is a major obstacle in developing a typing scheme for *P. putrefaciens* since unattacked strains are untypable. An extensive search for temperate phage in these strains was undertaken since lysogeny could be responsible for immunity to the phage isolated in this study. It was also hoped that phage carried by some of the other strains might be virulent for the 5 untypable strains, or that lethal bacteriocins might be produced which could be used as aids in identification.

Spontaneously released phages were not detected in broth cultures of any of the *P. putrefaciens* strains and attempts to induce the production of bacteriocins and/or temperate phage by brief heating and addition of H_2O_2 were uniformly negative. Induction with UV radiation and mitomycin C however, did yield one temperate phage. Both these treatments induced host strain P17 to release a phage which was virulent for only strain P11. No other temperate phage was detected and no bacteriocin-like reactions were ever observed.

DISCUSSION

The failure to obtain phage from sewage for all but one marine strain (Table 1) indicates that some degree of ecological specificity is involved with phage for this species since phages were readily obtained from sewage for the four terrestrial strains. The contamination of estuarine water by phages of terrestrial origin possibly accounts for the isolation of phages for the terrestrial strains from pier water.

The apparent low incidence of lysogeny among the strains used in this study may merely reflect a lack of suitable indicator strains. The observation of phage-like particles in one mitomycin C-induced lysate P19x of four strains examined by electron microscopy (Delisle, unpublished results), suggests that untypable strains may be lysogenic. Additional attempts to isolate phages for strains P4, P3, P8, P15, and P16 from other marine environments are necessary

TABLE

Host range of

Phage	Homologous host strain	Source	Isolation temperature	Lysis of S2	P2	Syl 1	8072	Syl 2	P19	8073	8071	P10
1	S2	Pier water	20 C	+	+	+	.	.	.	.	.	.
A	S2	Pier water	20 C	+	+	.	+	+	.	.	.	.
2	S2	Pier water	2 C	+	+	+	+	+	+	.	.	.
3	S2	Pier water	2 C	+	+	.	+	+	+	+	.	.
I	S2	Pier water	2 C	+	+	+	+	+	.	.	.	.
4	S2	Sewage	20 C	+	+	+	.	+	.	.	.	.
5	S2	Sewage	20 C	+	+	.	+	.	+	+	.	.
D	S2	Sewage	20 C	+	+	.	.	.	.	.	.	.
6	S2	Sewage	2 C	+	+	+	+	+	+	+	.	.
7	S2	Sewage	2 C	+	+	+	+	+	+	+	.	.
H	S2	Sewage	2 C	+	+	.	+	.	.	.	.	.
14	8071	Pier water	2 C	.	.	.	+	.	.	.	+	.
12	8071	Sewage	20 C	+	+	.	+	.	+	+	+	+
E	8071	Sewage	20 C	.	.	.	.	.	.	.	+	.
F	8071	Sewage	20 C	.	.	.	.	.	.	.	+	.
13	8071	Sewage	2 C	.	.	.	.	.	.	.	+	.
15[1]	8072	Sewage	20 C	+	.	.	+	.	.	.	.	.
16[1]	8072	Sewage	20 C	.	.	.	+	.	.	.	.	.
B	8073	Pier water	20 C	+	+	.	+	.	.	+	+	.
17	8073	Sewage	20 C	.	+	.	+	.	.	+	+	.
G	8073	Sewage	20 C	.	.	.	.	.	.	+	+	.
8	Syl 1	Pier water	20 C	+	+	+	.	.	.	.	.	.
C	Syl 1	Pier water	20 C	.	+	+	.	.	+	.	.	+
9	Syl 1	Pier water	2 C	+	+	+	.	.	.	.	.	.
10	Syl 1	Pier water	2 C	+	+	+	.	.	.	.	.	.
K	Syl 1	Pier water	2 C	+	.	+	.	.	.	.	.	.
11	Syl 2	Pier water	2 C	+	.	.	.	+	.	.	.	.
J	Syl 2	Sewage	2 C	.	.	.	.	+	.	.	.	.
18	P2	Pier water	20 C	+	+	.	.	+	.	.	.	.
25F	P2	Pier water	2 C	.	+	.	+	.	.	.	.	.
19	P6	Fish fillet	20 C	.	.	.	.	.	.	.	.	.
24	P6	Pier water	20 C	.	.	.	.	.	.	.	.	.
29	P10	Pier water	2 C	.	.	.	.	.	.	.	.	+
20	P11	Pier water	20 C	.	+	.	.	.	+	+	.	+
21	P11	Pier water	20 C	.	.	.	.	.	.	.	.	+
22	P12	Pier water	20 C	.	.	.	.	.	.	.	.	+
28	P12	Pier water	2 C	.	.	.	.	.	.	.	.	.
23	P13	Pier water	20 C	.	+	.	.	.	.	+	.	+
26F	P19	Fish fillet	2 C	.	.	.	.	.	+	.	.	.
27[2]	P19x	Pier water	2 C	.	.	.	.	.	.	.	.	+

[1]) Also lysed *P. fluorescens* strains 5.19 and 5.20, and *P. putida* 90 (biotype A)

[2]) No lysis at 20 C

1

40 *Pseudomonas putrefaciens* phages

P. putrefaciens test strains																		
P13	P17	P12	P14	P7	P1	P19x	P3	P20	P6	P21	P9	P21	P4	P5	P8	P15	P16
.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.
.	.	.	.	+	+	.	.	.	.	.	.	.	.	.	.	.	.
.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.
.	+	+	+	.	.	-	.	+	.	.	+	+	.	.	.	.	.
.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.
.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
.	.	.	.	+	+	.	+	.	.	.	.	.	.	.	.	.	.
.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.
.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
.	+	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.
.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.
.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.
.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
.	.	+	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.
.	.	.	.	.	.	.	.	.	.	+	+	.	.	.	.	.	.
.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
.	.	.	+	.	.	+	.	.	.	.	.	.	.	.	.	.	.

before it can be concluded that virulent phages for these strains do not exist in nature. The particular strains, for example, may have been picked up by the fish some distance from land so that phages for them may not be numerous in pier water.

Most of the phages isolated were capable of plaque formation at both 2 and 20 C, regardless of the temperature at which they were isolated. In almost all instances where plaques were formed at both temperatures, plaques were much larger at 2 C than at 20 C. Pre-growth temperature was found to have little effect on plaque formation on subsequent incubation, although some combinations of pre-growth and incubation temperatures prevented plaque formation in specific cases. One of these cases was investigated by conducting efficiency-of-plating tests, which showed phage No. 27 capable of producing plaques on the normal host P19 × at 2 C and unable to do so at 20 C. This type of behavior is quite unusual and is the subject of other studies now in progress.

This investigation was supported by Public Health Service Grant EF-00873-2 from the Division of Environmental Engineering and Food Protection.

Received 6 *June* 1968

REFERENCES

BLAIR, J. E. and WILLIAMS, R. E. O. 1964. Phage typing of staphylococci. ← Bull. World Health Org. **24:** 771-784.

BREED, R. S., MURRAY, E. G. D. and SMITH, N. R. 1957. Bergey's Manual of Determinative Bacteriology, 7th ed., p. 112. ← Williams & Wilkins Co., Baltimore.

CASTELL, C. H., RICHARDS, J. F. and WILMOT, I. 1949. *Pseudomonas putrefaciens* from cod fillets. ← J. Fish. Res. Bd. Can. **7:** 430-431.

CHAI, T., CHEN, C., ROSEN, A. and LEVIN, R. E. 1968. Detection and incidence of specific species of spoilage bacteria on fish. II. Relative incidence of *Pseudomonas putrefaciens* and fluorescent pseudomonads on haddock fillets. ← Appl. Microbiol. **16:** 1738-1741.

DERBY, H. A. and HAMMER, B. W. 1931. Bacteriology of butter. IV. Bacteriological studies on surface taint butter. ← Iowa Agr. Exp. Sta. Bull. **145:** 387-416.

DELISLE, A. L. and LEVIN, R. E. 1969. Bacteriophages of psychrophilic pseudomonads. I. Host range of phage pools active against fish spoilage and fish pathogenic pseudomonads. ← Antonie van Leeuwenhoek **35:** 307-317.

HUGH, R. and LEIFSON, E. 1953. The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various gram negative bacteria. ← J. Bacteriol. **66:** 24-26.

LEVIN, R. E. 1968. Detection and incidence of specific species of spoilage bacteria on fish. I. Methodology. ← Appl. Microbiol. **16:** 1734-1737.

LONG, H. F. and HAMMER, B. W. 1941. Distribution of *Pseudomonas putrefaciens*. ← J. Bacteriol. **41:** 100-101.