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Isolation of Psychrophilic Bacteriophage-Host Systems from Refrigerated Food Products[1]

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Thirty-eight bacteriophage-host systems were isolated from 22 of 45 refrigerated food products examined under psychrophilic conditions. Isolates were obtained from ground beef, pork sausage, chicken, raw skim milk, and oysters, whereas no isolations were made from liquid egg whites and processed meat products. Thirty of the 38 psychrophilic bacterial hosts were gram-negative rods, and 27 of these were classified within the genus *Pseudomonas*; three were members of the family *Enterobacteriaceae*. The remaining eight were gram-positive cocci, which were tentatively classified as *Leuconostoc*. Plate counts of psychrophilic bacteria were greater than 2.2×10^5/ml (g) in all but one sample which contained phage, whereas phage titers ranged from less than 100 to 6.3×10^6 plaque-forming units/ml (g). Phage isolates showed limited host ranges usually attacking only those hosts upon which they were isolated. Of eight phages tested against 13 cultures of known identity, one showed lytic action, and this was against strains of *P. fragi*.

Bacteriophages specific for psychrophilic species of the genus *Pseudomonas* have been reported (5, 13, 14). Isolations of these phages from food products have been made by Billing (2) and Delisle and Levin (5). Reports that streptococcal phage cause failure of acid development in the manufacture of cultured dairy products were first introduced in the mid-thirties. No literature reports were observed, however, regarding the occurrence of phages in association with their food spoilage hosts within refrigerated processed foods.

The purpose of this research was to determine the prevalence and concentrations of bacteriophage present within refrigerated foods. Isolated bacterial hosts were characterized as to their biochemical activities and were taxonomically classified.

MATERIALS AND METHODS

Bacteriophage enumeration. The agar layer method as described by Adams (1) was utilized. Plates were incubated at 20 C for 15 to 20 hr, after which plates showing 30 to 500 plaque-forming units (PFU) were counted with the aid of a Quebec colony counter.

Isolations of phage-host systems. All samples of ground beef, pork sausage, chicken parts, oysters, and processed meat products were purchased at local markets. Eleven-gram portions were blended for 2 min in 99 ml of phosphate-buffered distilled water, and the homogenate was plated with Standard Methods agar (19). Fresh, raw skim milk and egg whites were obtained from the Department of Food Science and Nutrition. Samples (11 ml) were diluted in 99-ml water blanks and then plated. Plates from all samples were counted after incubation at 10 C for 5 days (psychrophilic bacteria count/ml).

Twenty milliliters of the blended mixtures was centrifuged for 15 min at 15,000 × *g*. Membrane filtration (0.45-μm pore size) of the supernatant fluid followed. Skim milk samples were sterilized by adding 0.5 ml of chloroform to 9.5 ml of skim milk. Egg white, diluted 1:100, was passed consecutively through membrane filters of 3.0-, 1.2-, and 0.45-μm pore diameter. These preparations were labeled "food sample filtrates" and were used as sources of phages.

Representative isolated colonies were picked from plates on which psychrophilic bacteria of the foods had been enumerated. Colonies were chosen which differed in appearance under ultraviolet or incandescent light, or both. These were inoculated individually into tubes of Trypticase soy broth (TSB) and incubated at 20 C for 20 to 24 hr. One milliliter of each culture was transferred to a single tube containing 9 ml of TSB. To this was added 1 ml of the food sample filtrate from which the culture was isolated. After incubation for 10 to 12 hr, the presence or absence of phage, active against each isolate, was determined by the double-layer method of plating. Isolates which were lysed (discrete plaques) were twice restreaked to insure purity and then transferred to Trypticase soy agar (TSA) slants on which they were stored.

All isolates of the phage were thrice purified by picking single plaques with a toothpick into soft agar

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containing the host. Dilutions of this suspension were plated to obtain isolated plaques.

Phage titers in the food sample filtrates were determined by using, as indicators, strains of the isolates from the respective foods.

Identification of host isolates. All bacterial isolates were separated into two groups on the basis of the Gram stain reaction. Those which were gram-negative were further classified according to the results of the following tests: (i) colony morphology and pigmentation on TSA plates, (ii) motility by hanging drop mounts (17), (iii) type of flagellation by using the Leifson flagella stain (17), (iv) blood hemolysis on bovine blood-agar plates, (v) hydrolysis of casein on casein plates (3), (vi) hydrolysis of fat on spirit blue plates (6), (vii) production of fluorescent pigments on *Pseudomonas*-agar F (7), (viii) growth at various temperatures and modification of litmus milk in litmus milk tubes (8), (ix) breakdown of glucose in Hugh and Leifson stabs (9), (x) deamination of arginine in arginine broth tubes (8), (xi) type and extent of liquefication of gelatin in gelatin-agar stabs (15), (xii) reduction of nitrates in nitrate-peptone broth (15), (xiii) extent of acid production and the production of acetyl methyl carbinol in MR-VP broth (15), (xiv) presence of cytochrome oxidase by Kovac's oxidase test (10).

Most of these tests were also used in the classification of the gram-positive cocci. Other tests used to identify this group of organisms were as follows: (i) catalase production (8), (ii) detection of cytochrome system by the pseudocatalase test (4), (iii) growth in the presence of 6.5% NaCl, 0.1% methylene blue, *p*H of 9.6, at 10 and 45 C (8).

With the use of *Bergey's Manual of Determinative Bacteriology* and *Microbiological Methods* (3), these isolates were classified. Those organisms characterized as *Pseudomonas* were further subdivided into groups by the scheme of Shewan et al. (16).

Host range of phage isolates. The routine test dilution (RTD) was determined by the procedure of Adams (1) for all phages used in host range studies. Seven phage isolates were tested against 13 *Pseudomonas* cultures of known identity: five strains of *P. fragi*, three strains of *P. fluorescens*, two strains of *P. aeruginosa*, and one strain each of *P. ambigua* and *P. putrefaciens*. Dried TSA plates (10 cm diameter), seeded individually with these cultures, were spotted with each of the eight phages at their routine test dilutions. Plates were incubated at 20 C for 24 hr after which the susceptibility of the cultures to lysis was recorded.

All 38 phage isolates were tested for host range against each of the 38 host isolates. Plates (15 cm diameter) containing dried TSA were inoculated with 2 ml of a log-phase culture. The excess was poured off, and plates were allowed to dry at room temperature. A drop of the RTD of each phage, dispensed from a tuberculin syringe equipped with a 27-gauge needle, was placed on a designated area (1 through 38) of each dried, seeded plate. After incubation for 24 hr at 20 C, the plates were examined for areas of near confluent lysis. These experiments were repeated twice.

RESULTS AND DISCUSSION

Isolation of phage-host systems. Forty-five food samples yielded 216 bacterial isolates when plated at 10 C. As many as nine isolates were subcultured from some samples. More than one isolate of the same strain may have been picked due to the inability to differentiate organisms on the basis of colony morphology and fluorescence, the two criteria used.

Thirty-eight of the 216 isolates were lysed by phages present within their respective food sample filtrates. Twenty-eight of these were taxonomically different, and the remaining 10 formed five pairs which were taxonomically indistinguishable by the criteria used. Both members of each pair were from the same food sample. However, cross-reactions observed in the host range studies, discussed later, indicated that three of these pairs were definitely different, that one pair was probably different, and that one pair was the same.

Phages and their bacterial hosts were isolated from approximately 50% of the food samples (Table 1). This percentage, however, was likely biased by the unequal number of samples of each product examined. No phages were obtained from either egg white or luncheon meats, but all samples contained less than 3,000 psychrophilic bacteria per g. Phages were isolated only from products with high bacterial counts, and, except for one sample of pork sausage, all positive samples had psychrophilic bacteria counts in excess of 2.2×10^5/ml (g).

Phage titers in the food sample filtrates (Table 1) ranged from undetectable levels at a 1:100 dilution up to 6.3×10^6 PFU/ml. Evidently the added step in the isolation procedure, which involved enriching of cultures with the food sample filtrate, increased phage numbers in some samples so that after incubation a detectable concentration was present. Only one phage for each host was isolated from each filtrate, even though in some instances different plaque morphologies suggested that more than one phage was present. Thus, when the food sample filtrates were titered, the counts may have been due to more than one specific phage. Phage purification (plaque picking) was performed to eliminate contaminating phages which might have been present; this produced pure phage suspensions.

Identification of host isolates. Thirty-eight bacterial isolates were characterized. Thirty of these were gram-negative rods, whereas the other eight were gram-positive cocci. Twenty-seven of the former were classified as *Pseudomonas* by using the determinative scheme of Shewan et al. (16). All were oxidative. The six strains which

TABLE 1. *Phage and bacterial content of refrigerated foods*

Sample	No. of samples examined	No. of samples yielding phage	No. of phage isolated	Ranges for samples yielding phage	
				PBC[a]/g (ml) (× 10^5)	PFU[b]/g (ml) (× 10^3)
Ground beef	17	11	16	2.2– 280	<0.1– 9.4
Pork sausage	7	4	8	0.9–8,000	<0.1– 340.0
Chicken[c]	8	4	9	120.0–9,000	<0.1–6,300
Raw skim milk	5	2	2	5.0– 10	ND[d]
Oysters	1	1	3	4.5	<0.1–1,000
Egg white	2	0	0		
Luncheon meats	5	0	0		
Totals	45	22	38		

[a] Psychrophilic bacteria count.
[b] Plaque-forming units.
[c] Sample from skin of fryer part.
[d] Not determined.

produced diffusible pigment on *Pseudomonas* agar F were placed in group I, and the remaining 21 fell in group II. The three other strains of gram-negative rods were classified into the family *Enterobacteriaceae* without further differentiation as to genus.

Comparison of the two groups of *Pseudomonas* showed definite differences. Group I was much more biochemically active with all isolates hydrolyzing fat and specific proteins, whereas a lower percentage of the isolates within group II showed this ability. Their optimum temperature was 21 C or lower; thus, these isolates showed truly psychrophilic, rather than mesophilic, temperature ranges of growth. However, all of these organisms were isolated from plates incubated at 10 C.

It was somewhat surprising that 27 of these 30 isolates were members of the genus *Pseudomonas* even though this genus constitutes a large percentage of the psychrophilic isolates from refrigerated foods (21). Phages for other genera apparently were not present.

All eight isolates of gram-positive cocci were shown to be very similar in their biochemical activities. They occurred in chains, dividing in one plane. Their colonies on blood-agar were typical of streptococci in appearance but showed no hemolysis. They were slightly catalase positive (8), but the test of Deibel and Evans (4) indicated that pseudocatalase produced the reaction. Because acid production was not observed in litmus milk and the cultures were nonhemolytic, these isolates were not considered to be members of the genera *Aerococcus* (20) or *Streptococcus*. Their growth on blood-agar under aerobic conditions was not typical of *Pediococcus*, nor was their chain formation.

They fermented glucose and grew in 6.5% NaCl, in broth (*p*H 9.0), and at 10 but not at 45 C. Therefore, they were tentatively classified as an unknown species of *Leuconostoc*.

TABLE 2. *Cross-reactions of phages with host bacteria isolated from foods*[a]

Bacteriophages	Hosts
Pseudomonas group I	
k8	B4, K8
Pseudomonas group II	
b2a	B2a, B2b, B5, I
b2b	B2a, B2b, B5
b5	B2a, B2b, B5
i	B2a, I
u3	B2a,[b] B2b,[b] U3, V3
v3	U3, V3
Enterobacteria	
e2	E2, E3
e3	E2, E3
Leuconostoc	
d4	D4, M2

[a] All other phages reacted only with their homologous host.
[b] Less than 10 plagues.

Host range studies. Seven of the phages isolated from the food samples (phages ps1, wy, i, d, f, y2, and gb3) were tested for ability to lyse 13 cultures of known identity. The purpose was to gather additional information as to the identity of the hosts found in the food samples. Only phage i lysed any of these bacteria, and it lysed strains 7, P27, and K1 of *P. fragi*. The hosts of three other phages Ps1, wy, and y2, were identified as *P. fragi*, but these phages failed to lyse any of the *P. fragi* cultures. Phage strain f, from *P. fluorescens*, failed to lyse any of three known strains of the same species.

Some *Pseudomonas* and *Xanthomonas* phages

have been shown to react across species and even generic lines (15, 18), whereas other phages of *Pseudomonas* were species to strain specific (11, 12). Eleven of our *Pseudomonas* cultures, representing four of the five species, are common food contaminants. Therefore, it was not unlikely that these 11 strains would react with the isolated phages. The phages tested may well have been strain specific, and, since their homolgous strains were not present, lysis was not observed.

The 30 gram-negative hosts and their respective phages were cross-reacted to determine host ranges (Table 2). All phages produced confluent lysis on their homologous hosts, but few attacked any other bacterial strain. The greatest number of phages which showed cross-reactivity, 3, was found within the B series, all isolated from a single, ground beef sample. Based on biochemical studies, only hosts B2b and B5 appeared alike, and these were believed to be different strains as shown by phage susceptibility (phage typing).

There was little cross-reactivity between phages and bacteria isolated from different samples, although some phages showed the ability to lyse isolates from other samples, viz. k8 on B4; b2a on I; i on B2a; u3 on B2a, B2b, and V3; and v3 on U3. Based on taxonomic data, hosts B2b and I appeared to be the same species (from different samples); this was further substantiated by the host range studies. These tests verified other taxonomic data, showing that practically all the bacterial isolates were at least different strains.

Phage b2a lysed cultures B2a, B2b, B5, and I. These isolates were all classified within Group II of Shewan et al. (17). Tentative taxonomic classification of three of these four isolates as *P. fragi* had been made independently. Nine other gram-negative isolates were also classified as *P. fragi* but were not lysed by phage b2a. Phage b4, besides lysing its homologous host B4, showed confluent lysis on culture K8. These hosts were found to belong to Group I. No phage was shown to cross-react between groups.

Of the phages to the gram-positive cocci, only phage d4 cross-reacted, lysing host M2. Host range studies were performed before the bacterial hosts were characterized; therefore, all isolates of gram-positive cocci were tested with phages specific for gram-negative hosts, and vice versa. As would be expected, no cross-reactions were observed between these two groups.

In conclusion, it is clear that phage-host systems exist in refrigerated food products. It appears that both phage and host are present as food contaminants, and that these phages enter either as prophages within lysogenic hosts or as free, virulent phages.

At present, work is in progress to determine what effect these phages have upon their food spoilage hosts within foods. These phage-host systems may significantly influence the shelf life of refrigerated food products.

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