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Susceptibility of Psychrotrophic Pseudomonads of Milk Origin to Psychrotrophic Bacteriophages

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A total of 47 psychrotrophic pseudomonads isolated from raw milk came from Newfoundland (19 isolates), British Columbia (6 isolates), Ontario (19 isolates), and Cork, Ireland (3 isolates). The susceptibility of these was tested against 30 bacteriophages isolated from cold-storage beef. Distinct lysotypes were observed with isolates representing different geographic regions. Phages as agents in the control of bacterial populations in milk is discussed.

Psychrotrophic pseudomonads that produce extracellular heat-stable proteases represent a biochemically diverse group that is not easily defined taxonomically. Bacteriophage-typing schemes have been used to study human pathogens (14) and phytopathogens (2, 12). Pseudomonads sensitive to phages have been isolated from different environments (5, 13, 18). Phages capable of infecting host bacteria at low temperatures (0 to 7°C) have been described previously (9, 13, 19) and are termed psychrotrophic phages.

Food-spoiling psychrotrophic pseudomonads in refrigerated foods (4) excrete heat-stable lipases (7) and proteases (8, 15). The susceptibility of psychrotrophs of milk origin to phages has been examined for the first time in this investigation. An attempt was made to classify these bacteria on the basis of their cross-sensitivity to phages. In addition, further characterization of 19 isolates from Newfoundland was carried out, using biochemical tests to determine if these, in conjunction with the phage typing, would offer their identification.

The 19 isolates from Newfoundland (15) when analyzed by the scheme of Shewan et al. (17) fell under groups I and II. The majority of the isolates belonged to group I, comprising fluorescent pseudomonads, and only four isolates (T3, T13, T15, and T24) belonged to nonfluorescent pseudomonads of group II. The biochemical tests separated these isolates into 17 distinct biotypes. The biochemical tests included utilization of carbohydrates (arabinose, dextrose, xylose, mannose, galactose, and malibiose), sensitivity to antibiotics (streptomycin, chloromycetin, terramycin, and penicillin), gelatin hydrolysis, ability to grow on salmonella-shigella plates, and detection of arginine dihydrolase in these isolates. All tests were negative for mannose and malibiose, all isolates were resistant to 3 IU of penicillin, and all hydrolyzed gelatin. Biochemical tests such as these may be useful in addition to other proposed tests (3, 10, 16, 17) for the precise identification of psychrotrophic pseudomonads.

The biochemical diversity of these pseudomonads defies taxonomic classification beyond the species level. However, the following results indicate that these bacteria can be differentiated on the basis of a distinct pattern of susceptibility to phages (i.e., lysotypes). Psychrotrophic pseudomonads and their homologous phages used in this investigation have been described already (9). A phage titer averaging 10^7 PFU/ml was obtained by this method. Phage stocks were stored over chloroform (4%) at 4°C. Phage titration was performed by the method of Adams (1), using the agar overlay technique. The susceptibility of psychrotrophic pseudomonads was also tested by using spot tests. The plates carrying a lawn of host cells were spotted with a loopful of phage suspension. The plates containing phages and infected bacteria were incubated at either 25°C for 24 h or 7°C for 7 to 10 days. The results obtained by incubation at these temperatures were comparable.

In the spot tests a single plate was spotted with eight different phages. A clear zone of lysis indicated susceptibility of the host cells. At least three attempts were made to determine the sensitivity of psychrotrophs to the phages. Although the phage sensitivity at different phage dilutions was examined, 10^7 PFU/ml was used as the routine test dilution.

When susceptibility of all 47 pseudomonads to a specific phage was examined, a distinct pattern appeared (Table 1). Phage CM20 exhibited the broadest specificity, being able to infect 42.6% of the pseudomonads, while phage C25 showed the second highest infectivity at 36.1%. Seven phages (23.3%) were unable to infect any of the isolates. Phages infecting isolates from Newfoundland, Ottawa, British Columbia, and Ireland were different.

Table 2 lists lysotypes for each isolate. A total of 25 distinct lysotypes were recognized. Of the 12 distinct lysotypes obtained with isolates from Newfoundland, the majority contained more than one phage per lysotype except in the cases of isolates T10, T13, and T15, which were lysed by a single phage. The percentage of bacterial isolates susceptibile to lysis by any one phage ranged from 3.3 to 33.3% in the case of isolates from Newfoundland and from 3.3 to 43.3% in the case of the remaining psychrotrophs.

Of the 19 pseudomonads from Ottawa, only 3 (*Pseudomonas fluorescens* 15, 49, and S63) were found resistent to all phages tested. The remaining susceptible isolates could be differentiated by 10 distinct lysotypes. Phage C27 infected isolates P26, W, and 52, while phage C9 was active on isolates 0-0, 2-1, and 2-2. The isolates from British Columbia were susceptible to phages C9 and C33 (Table 2).

Several strains of mesophilic bacteria were also tested against the phages. However, lysis obtained with these bacteria can at best be described as very weak when compared with that caused when psychrotrophic pseu-

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TABLE 1. Susceptibility of psychrotrophic pseudomonads to phage[a]

Phage	Susceptible pseudomonads[b]	% Susceptible
C2	T2, T18, T19, T22, T25, T26, T27, T28, 13, 32A, 67, 73, 97, 192, 240, C15456, AFT-7, AFT-21, AFT-36	40.4
C3	13, 32A, 33, 67, 73, 97, C15456	14.9
C6	13, 32A, 192, 240, C15456, AFT-7, AFT-21, AFT-36	17.0
C8	13, 73, 192, C15456	8.5
C9	73, 0-0, 2-1, 2-2, 8-1, 6-3, 8-2	15.9
CM13	T1, T6, T8, T25, T22	10.6
C14	33, 73, 192, C15456	8.5
C15	T6, T8, T19	6.4
C16	T8	
C19	T1, T6, T12, T18, T22, T25, T27	14.9
CM20	T1, T2, T3, T6, T8, T10, T12, T13, T15, T16, T18, T19, T20, T22, T24, T25, T26, T27, T28, C15456	42.6
C21	73, C154561	4.3
C25	T1, T2, T3, T12, T16, T18, T19, T20, T22, T24, T25, T26, T27, T28, AFT-7, AFT-21, AFT-36	36.2
C26	13, 192, C15456	6.4
C27	91, W, B52, M5, 22F, B34	12.7
C28	13, 32A, 33, 192, C15456	10.6
C29	T8, T20, C15456	6.4
C31	T2, T8, 91, B52, M5, 22F, B34	14.9
C32	T2	2.1
C33	8-1, 8-2, 6-3	6.4
C34	C15456	2.1
C35	T1	2.1
C37	T20, T22, T25, 73, C15456, 91, B52, M5, 22F	19.1

[a] Phages and their homologous hosts are listed in Table 2.
[b] A total of 46 pseudomonads were tested. Isolates from Ireland (AFT-7, AFT-21, and AFT-36), British Columbia (0-0, 2-1, 2-2, 8-1, 6-3, and 8-2), Newfoundland (numbers with letter T), and Ontario (remaining) were included in the test. C15456 represents ATCC 15456.

TABLE 2. Phage sensitivity[a] pattern of psychrotrophic pseudomonads from Newfoundland

Pseudomonas sp. isolate(s)	Lysotype	% Phage infection[b]
T10, T13, T15	CM20	3.3
T3, T16, T24	C20, C25	6.6
T26, T28	C2, CM20, C25	10.0
T6	CM13, CM15, C19, CM20	13.3
T12	C15, C19, CM20, C25	13.3
T18, T27	C2, C19, CM20, C25	13.3
T19	C2, C15, CM20, C25	13.3
T20	CM20, C25, C29, C37	13.3
T1	CM13, C19, CM20, C25, C35	16.6
T2	C2, CM20, C25, C31, C32	16.6
T22, T25	C2, CM13, C19, CM20, C25, C37	20.0
T8	CM13, C15, C16, C19, CM20, C29, C31	23.3
240	C6	3.3
0-0, 2-1, 2-2	C9	3.3
P26, W, B52	C27	3.3
67, 97	C2, C3	6.6
B34	C27, C34	6.6
8-1, 6-3, 8-2	C9, C33	6.6
AFT-7, AFT-21, AFT-36	C2, C6, C25	10.0
91, M5, 22F	C27, C31, C37	10.0
32A	C2, C3, C6, C28	13.3
33	C2, C3, C14, C28	13.3
73	C2, C3, C8, C9, C14, C21, C37	23.3
13, 192	C2, C3, C6, C8, C14, C26, C28	23.3
C15456	C2, C3, C6, C8, C14, C21, C22, C26, C28, C29, C34, C37, CM20	43.3

[a] Bacterial lysis was determined by spotting a loopful of phages on a lawn of bacteria; plates were incubated at 25°C for 24 h.
[b] A total of 30 psychrotrophic phages were used. Values represent the percentage of the 30 phages able to lyse each strain. C15456 represents ATCC 15456.

domonads were hosts. The mesophilic strains tested included both gram-negative and gram-positive bacteria. The majority of the nonpseudomonad strains were weakly infected by phages C6, C8, CM20, C25, and C26. *P. aeruginosa*, *P. fluorescens*, and *P. marina* were easily separable from one another by weak but distinct lysotypes.

There was no correlation between the lysotypes obtained by phage sensitivity tests and the biotypes obtained by the biochemical tests. Thus, psychrotrophic pseudomonads of milk origin are similar to those from refrigerated foods (9, 18) in their biochemical variability.

The pseudomonads used in the current study were all of milk origin and are sensitive to attack by psychrotrophic phages. Phages active against other pseudomonads (4, 5, 9, 13, 18) have been reported. This and other reported work (5, 6, 9, 18) lad to a common conclusion that psychrotrophic pseudomonads comprise a relatively large number of distinct strains which are difficult to differentiate by biochemical tests.

The possible application of a phage collection for a biological control of food-spoiling pseudomonads remains to be studied. The shelf life of milk and milk products may be extended either by delay of onset of growth in the psychrotrophs contaminating milk or by limiting their growth by lysis. Greer (9) has shown that phages could delay the onset of *Pseudomonas* sp. growth by up to 6 days at 7°C. The use of phages for the biological control of psychrotrophic pseudomonads could lead to the selection of phage-resistant strains, and this is an important factor to bear in mind. It may be necessary to use a mixture of different phages to achieve maximum lysis of food-spoiling bacteria.

In conclusion, all phages appeared to be host specific since only pseudomonad strains were clearly lysed in the spot test. The nonpseudomonad strains exhibited only weak reactions. No one phage could lyse all 47 pseudomonads and conversely no one bacterial isolate was lysed by all 30 phages. Whether different bacterial strains that are infected by distinct lysotypes should be ultimately considered as distinct species, subspecies, varieties, biotypes, or host types remains a matter of opinion and a problem for further study. It is interesting to note that pseudomonads from different geographic regions are identifiable by distinct lysotypes. The majority of isolates from Newfoundland were lysed by phages C2, CM13, C19, CM20, and C25, while those from British Columbia were susceptible to phages C9 and C33. The isolates from Ireland were infected by phages C2, C6, and C25 and those from Ottawa were susceptible to phages C2, C3, C6, C14, C26, C28, C31, and C37. The isolates from Newfoundland are separable by 12 distinct lysotypes, while those from Ottawa are separable by 10 different lysotypes. The isolates from Vancouver were distinguishable by two lysotypes (C9 and C33) and those from Ireland were distinguishable by a single lysotype (C2, C6, and C25).

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