APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1993, p. 2914–2917
0099-2240/93/092914-04$02.00/0

Vol. 59, No. 9

Biological Inactivation of Adhering *Listeria monocytogenes* by Listeriaphages and a Quaternary Ammonium Compound

B. ROY,[1,2] H.-W. ACKERMANN,[3] S. PANDIAN,[1,2] G. PICARD,[2] AND J. GOULET[1,2]*

Dairy Research Center (STELA), Faculty of Agriculture,[1] Félix d'Hérelle Reference Center for Bacterial Viruses, Department of Microbiology, Faculty of Medicine,[3] and Department of Food Science and Technology, Faculty of Agriculture,[2] Université Laval, Ste-Foy, Québec, Canada G1K 7P4*

Received 22 February 1993/Accepted 10 June 1993

The use of listeriaphages as a means of disinfecting contaminated stainless-steel and polypropylene surfaces was investigated. Surfaces artificially contaminated with *L. monocytogenes* 10401 and 8427 were sanitized with suspensions of listeriaphages (H387, H387-A, and 2671), all belonging to the *Siphoviridae* family. Phage suspensions at concentrations of up to 3.5×10^8 PFU/ml were at least as efficient as a 20 ppm solution of a quaternary ammonium compound (QUATAL) in reducing *L. monocytogenes* populations. A synergistic activity was observed when two or more phages were used in combination and when phages were suspended in QUATAL. The biological activity of the three phages was not affected by QUATAL concentrations of 50 ppm and a contact time of 4 h.

Listeria monocytogenes is a ubiquitous bacterium (8) that is found not only in raw food but also on working surfaces of food-processing plants. Processed foods may thus be contaminated by *L. monocytogenes* even if the raw ingredients are free of the pathogen. This is thought to be the main source of *L. monocytogenes* in processed foods, and therefore contaminated surfaces play an important role in the transmission of food pathogens.

The recurrence of *L. monocytogenes* in food plants is a major problem and has even led to the closing of some plants in the past (12). The use of efficient disinfectants and good manufacturing practices is thus mandatory for preventing pathogenic contaminations. The resistance of surface-adhering *L. monocytogenes* cells to most chemical disinfectants under specific conditions (9) highlights the importance of developing new disinfection approaches and more efficient sanitizing agents. Since higher concentrations of chemical disinfectants would be needed to overcome the resistance of adhering *L. monocytogenes* cells, this would increase their burden on the environment and constitute a health hazard for employees. The development of specific biodisinfectants consisting of an aqueous suspension of listeriaphages would obviate these inconveniences and would offer new alternatives in fighting food-borne pathogens.

The use of bacteriophages for decontaminating food plant surfaces (16) and even food itself (13) represents an innovative and promising approach to the problem of microbial food contamination. Because of their high specificity (23), bacteriophages should have minimal impacts on the microbial ecology of foods, processing plants, and the environment. In this study, we investigated many formulations of phage suspensions to improve the level of destruction of *L. monocytogenes*. Phages were used in suspensions of a single phage, in mixtures of different phages, or in combination with a chemical disinfectant. The aim of this study was to evaluate the efficacy of listeriaphages in sanitizing stainless-steel (SS) and polypropylene (PP) surfaces contaminated with *L. monocytogenes*. The overall objective was to develop a biodisinfectant formulation specifically active against *L. monocytogenes* and capable of preventing its development in food and food-processing plants.

* Corresponding author.

MATERIALS AND METHODS

Bacteria, phages, and media. The two strains of *L. monocytogenes* used (10401, serovar 4, and 8427, unknown serovar) were from the Félix d'Hérelle Reference Center for Bacterial Viruses. Both strains were maintained on TSY agar (Trypticase soy agar [BBL, Cockeysville, Md.], 0.6% yeast extract [Difco, Detroit, Mich.]). Colonies were picked and inoculated in 250-ml flasks containing 100 ml of CYLG medium. This medium contained 2 g of glucose, 0.02 g of $MgSO_4$, 0.02 g of $MnSO_4$, 0.02 g of $FeSO_4$, 0.02 g of $CaCl_2$, 10 g of tryptone (Difco), 5 g of yeast autolysate (Difco), 10 ml of 50% potassium lactate, and H_2O to 1,000 ml. The undissolved materials were filtered through Whatman no. 3 filter paper before autoclaving.

The three listeriaphages used (2671, H387, and H387-A) were from the phage collection of the Félix d'Hérelle Reference Center. They belonged to the *Siphoviridae* family, characterized by long, noncontractile tails (2). The phages were selected for their wide host range as determined on *Listeria* strains from our collection. Phage H387-A is a derivative of phage H387 (22) propagated on *L. monocytogenes* IP31-A, a *hem* mutant of strain IP31. The titers of viral particles were determined on agar surfaces. Phage stocks ($>3 \times 10^8$ PFU/ml) were stored at 4°C for no longer than 2 weeks prior to use.

Test surfaces. SS and PP cylinders (outside diameter, 8 mm; inside diameter, 6 mm; length, 10 mm [as described in reference 11]) were sterilized in a 0.1% asparagin solution (Difco) and rinsed with sterilized water. The cylinders were contaminated by immersion for 60 min at 26°C into fresh cultures of *L. monocytogenes* (3×10^8 CFU/ml) and air dried aseptically by being placed vertically over two sterilized filter papers (Whatman no. 3) in a petri dish for 30 min at 37°C.

Disinfection procedure. After being dried, contaminated cylinders were first rinsed as follows. The cylinders (3×15 per tested bacteria) were rinsed in physiological saline (300 ml of 0.8% NaCl) for 2 min under mild agitation. The

TABLE 1. Variance analysis and interactions as function of sampling method and date (F values)

Source[a]	df[b]	$P > F$[c]
Bacteria	1	0.0100 (*)
Chemical product	3	0.0001 (*)
Phages	3	0.0001 (*)
Phages * Cyl	3	0.0376 (*)
Bact * Cyl (adhesion)	1	0.7900 (NS)
Bact * Cyl (biological treatments)	1	0.0202 (*)
Chem * Phages	9	0.0001 (*)
Phages * Chem * Cyl	9	0.0028 (*)

[a] Abbreviations: Phages * Cyl, interaction between listeriaphages and cylinders; Bact * Cyl, interaction between bacteria and cylinders; Chem * Phages: interaction between chemical product and listeriaphages; Phages * Chem * Cyl: interaction between listeriaphages, chemical product, and cylinders.

[b] df, degrees of freedom.

[c] *, $P < 0.05$; NS, no significant difference. For these determinations, $n = 3$.

cylinders were dried as above and then transferred into 5 ml each of the following decontaminating solutions: (i) pure suspensions of each phage, (ii) a mixture of equal quantities of the three phages, (iii) 1 to 100 ppm solutions of QUATAL, and (iv) a combination of phages and QUATAL (1:1, vol/vol). According to the manufacturer (Ecochimie Ltée, Ste-Julie de Verchères, Quebec, Canada), the active ingredients of QUATAL are 10.5% *N*-alkyldimethyl-benzylammonium-HCl (40% C-12, 50% C-14, 10% C-16) and 5.5% glutaraldehyde. After a decontamination period of 1 h, each cylinder was rinsed with sterile 0.1% peptone solution, placed in 5 ml of the same solution, and vortexed vigorously for maximal release of adhering cells. Recovered cells were enumerated by plating on TSY agar.

Resistance of phages to QUATAL. Phages were suspended in solutions of QUATAL (1 to 100 ppm) for 4 h. Samples were taken every hour for phage titer determination; QUATAL was neutralized with 20% (vol/vol) lecithin (Le Naturaliste J.M.B., Boucherville, Quebec, Canada) before plating on TSY agar. Phage titers were determined on a culture of *L. monocytogenes* IP31.

Statistical analysis. All microbial counts were transformed to $\log_{10}$ values, and variance analysis ($\alpha = 0.05$) was carried out by following a random-block design, using a general linear-model procedure (program GLM; SAS Institute, Inc., Cary, N.C.). Homogeneity of variance was verified by the Levene test (21). The normality of residues was tested by the Shapiro-Wilk test and the Q-Q plot, and then different parameters were compared by using the LSD (least statistical difference) test. The experiments were carried out in triplicate. Results of the statistical analysis are shown in Table 1.

RESULTS AND DISCUSSION

Phage host range. The testing of *Listeria* strains (*L. monocytogenes* 5290, 10401, IP31, 8427, and 1089 and *L. ivanovii*) for killing by listeriaphages is clearly the first step in a biocontrol strategy—such as proposed here—in order to select a phage with a wide spectrum. Each of the three phages, H387, H387-A, and 2671, had previously been tested in our laboratory against eight *Listeria* strains (obtained from Health and Welfare Canada and from Fisheries and Oceans, Ottawa, Canada) for phage typing (data not shown). Phages H387 and H387-A lysed all strains, whereas phage 2671 lysed

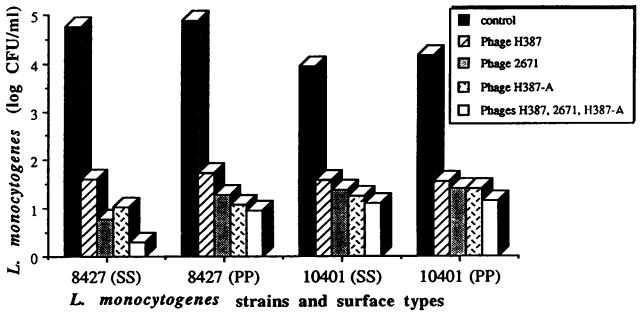

FIG. 1. Recovery of adhering *L. monocytogenes* cells from contaminated SS and PP surfaces before and after exposure for 1 h to listeriaphages (3.5×10^8 PFU/ml).

50% of them. Because of their wide host range, listeriaphages may be used as efficient biodisinfectants against adhering *L. monocytogenes* organisms. The detection of *Listeria* species by phage typing has already been suggested as a test of the recurrence of this microorganism in food-processing plants (15).

Adherence of *L. monocytogenes* to surfaces. The adherence of all tested strains of *L. monocytogenes* to PP surfaces was slightly greater than to SS surfaces (4.25 ± 4.04 and 4.00 ± 3.79 log CFU/ml, respectively, for strain 10401, and 4.99 ± 4.84 and 4.93 ± 4.96 log CFU/ml, respectively, for strain 8427). However, these differences were not statistically significant ($P = 0.79$). On the other hand, strain 8427 adhered in larger numbers than strain 10401 to all surfaces ($P = 0.01$). The adhering behavior has already been reported and related to factors such as free energy (5), hydrophobicity of contact materials, and ionic interaction between bacteria and surfaces (10). Adhesion of bacteria to surfaces can also involve production of exopolymers (glycocalyx) by microorganisms (7). According to Mafu et al. (20), the interfacial free energy of adhesion of *L. monocytogenes* Scott A is lower for PP surfaces than for SS surfaces. Since free-energy calculations predict that PP should allow for better attachment of microorganisms than SS does (19, 20), one might presume that sanitation of SS surfaces should be easier than for PP surfaces. Indeed, several authors (14, 24, 25) have reported more difficulties in disinfecting plastics than other types of solid surfaces. Because of the presence of many scratches (20), soft surfaces offer more room to the cells for protection against mechanical and chemical stresses.

Disinfection by listeriaphage suspensions. When single-listeriaphage suspensions were used to sanitize SS and PP surfaces, the destruction level was about 3.4 log units. The efficiencies of phages H387, H387-A, and 2671 were about equal in inactivating *L. monocytogenes* 10401 and 8427 on PP and SS surfaces. However, strain 8427 was more easily inactivated on SS than on PP with the mixture of three phages ($P = 0.02$). In fact, the statistical analysis showed an interaction between the strains and the cylinders. The efficiency of the single phages did not seem to be influenced by the nature of the solid surfaces. However, the mixture of the three phages showed a better efficiency on SS than on PP ($P = 0.04$). Results in Fig. 1 suggest a synergistic effect of phages 2671, H387, and H387-A when used in combination for disinfecting SS surfaces. The destruction level for the mixture of all three phages was 3.5 log units on PP compared with 3.7 log units on SS. Reduction levels of 99 to 99.9% in bacterial cell counts were thus observed with listeriaphages,

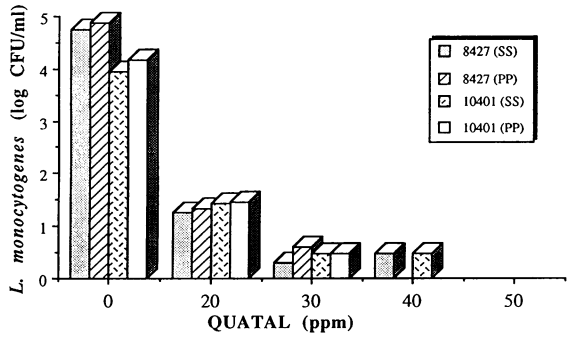

FIG. 2. Recovery of *L. monocytogenes* 8427 and 10401 from contaminated SS and PP surfaces after exposure for 1 h to several concentrations of QUATAL.

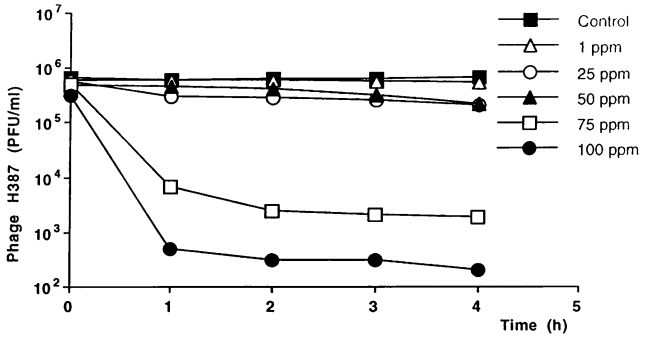

FIG. 4. Resistance of phage H387 to QUATAL (1 to 100 ppm). Phage were suspended in various concentrations of QUATAL for periods of up to 4 h. Aliquots were withdrawn hourly and neutralized with lecithin, and the titer was determined.

but these will have to be improved to meet the recommended reduction level of 99.999% in a 30-s exposure for a chemical sanitizing agent (6).

The development of resistance against bacteriophages is a well-known phenomenon (1, 3). It can be minimized or prevented by using mixtures of two or more phages differing in their mode of infection of bacterial cells. Similarly, the use of listeriaphages in combination with a chemical sanitizer may improve the level of destruction of *L. monocytogenes* through a synergistic effect. It appears very unlikely that a single strain could develop resistance to more than one inhibitory substance simultaneously.

Mixed disinfecting solutions. A 50 ppm concentration of QUATAL was required for total destruction of all strains on SS and PP surfaces (Fig. 2). The effects of chemical and biochemical treatments were dependent of the concentrations used ($P = 0.0001$). The mixture of three listeriaphages at concentrations up to 3.5×10^8 PFU/ml was about as efficient as a 20 ppm solution of QUATAL in sanitizing contaminated surfaces (Fig. 1 and 2). The combination of a biological and a chemical sanitizer thus appears more efficient than the use of either one individually ($P = 0.0001$). The addition of listeriaphages to a quaternary ammonium compound solution allowed us to reduce the concentration of chemicals to as little as 40 ppm for destroying the whole population of contaminating *Listeria* cells (Fig. 3). The listeriaphage-QUATAL suspension was more efficient in inactivating *Listeria* cells on SS than on PP ($P = 0.0028$). Indeed, better disinfection efficiencies with chemical products had already been reported for SS than plastics for several species of bacteria (25). However, one must bear in mind that the efficiency of chemical compounds may also be influenced by environmental factors such as pH, organic residues, water hardness, and temperature (4). The combination of a chemical agent with bacteriophages appears to be a means of reducing the concentration of environmentally hazardous compounds while maintaining the same disinfecting activity against *L. monocytogenes*. However, this can be accomplished only with chemicals that will not inactivate bacteriophages.

Exposing listeriaphages (H387) to various concentrations of QUATAL (1 to 50 ppm) for up to 4 h did not destroy their infectivity (Fig. 4). However, at concentrations of 75 and 100 ppm, phage titers were reduced by 3 to 4 log units within 1 h. Other listeriaphages (2671 and H387-A) showed a similar sensitivity (data not shown). This work has shown that listeriaphages (at concentrations of 3.5×10^8 PFU/ml) may be as efficient as a 20 ppm solution of QUATAL in sanitizing contaminated SS and PP surfaces. This performance has to be improved to reach a destruction efficiency comparable to that of 50 ppm of QUATAL (100% destruction). The observed synergistic effect of listeriaphages and QUATAL shows a promising avenue in improving the destruction level. Further work is being carried out to optimize physical and chemical conditions and hence to improve the efficiency of this new approach of biodisinfection. The recent isolation of a new, highly virulent listeriaphage (A511) by Loessner et al. (17, 18) could help in the development of new formulations of improved efficacy.

Although listeriaphages have shown a good decontamination activity against two strains of *L. monocytogenes*, further investigations are currently being carried out to improve their infectious and lytic activities and increase phage yields by propagation in liquid media.

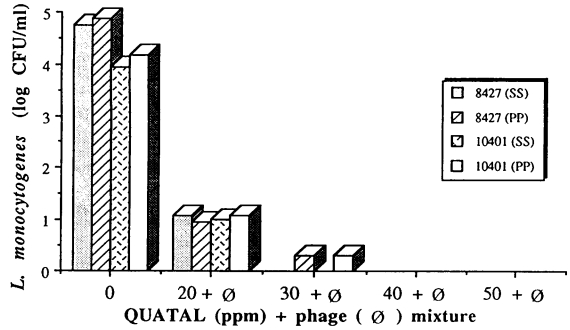

FIG. 3. Recovery of *L. monocytogenes* 8427 and 10401 from contaminated SS and PP surfaces after exposure for 1 h to a suspension of listeriaphages (10^8 PFU/ml) mixed with several concentrations of QUATAL.

ACKNOWLEDGMENTS

We thank Fisheries & Oceans Canada, AQUIP and Alpha-Biotech Inc., Quebec, for providing financial assistance for this study.

We thank EcoChimie, Quebec, for providing the chemical disinfectants used in this project. We thank Steve Méthot for providing statistical assistance.

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