13 Phage Therapy in Animals and Agribusiness

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13.1. INTRODUCTION

Since the discovery of bacteriophages in 1915–1917, they have been used to prevent and treat various bacterial infections. Although *phage therapy* has been historically associated with the use of bacteriophages in human medicine, phages also have been extensively used in veterinary medicine and in various agricultural settings. The history and various aspects of phage therapy in humans are reviewed in Chapter 14 of this book. In this Chapter, we review the past and current use of phages to prevent and treat naturally occurring and experimentally induced infections of animals. In addition, we discuss the potential applications of phage therapy in various agricultural settings, including the potential value of bacteriophages for improving the safety of foods and preventing foodborne diseases of bacterial etiology, and their potential to reduce the use of antibiotics in livestock.

13.2. USING PHAGES TO PREVENT AND TREAT ANIMAL INFECTIONS

13.2.1. The First Known Use of Phages to Treat Bacterial Diseases of Animals

The first-known therapeutic use of phages in veterinary medicine is associated with Felix d'Herelle, the co-discoverer of bacteriophages. In the spring of 1919, large outbreaks of lethal fowl typhoid in chickens occurred in the Acris-sur-Aube region of France. D'Herelle analyzed several dead animals from the outbreaks, and he isolated and identified *Salmonella gallinarum* as the etiologic agent of the disease. D'Herelle also isolated bacteriophages from the chickens, and he examined their efficacy in preventing and treating *S. gallinarum* infections in six experimentally infected chickens (d'Herelle, 1921, 1926). The results of the study were promising: Phage administration prevented the birds from succumbing to the bacterial infection, whereas the two control chickens not treated with phages died after a single dose of the challenge strain.

The promising results of the small pilot study prompted d'Herelle to almost immediately initiate larger trials, which he called *immunization experiments*. One hundred chickens were infected with *S. gallinarum* and 20 of them were treated ("immunized") with *S. gallinarum*-specific phage. The 20 phage-treated birds survived; whereas 60 (75%) of the phage-untreated birds died. Encouraged by these results (and the results of similar studies with rabbits and buffalo), d'Herelle subsequently used other phages in his collection to treat bacterial dysentery of humans (see Chapter 14). Also, the results of d'Herelle's studies examining phage prophylaxis and treatment of fowl typhoid prompted other investigators to begin examining the utility of bacteriophages in preventing or treating various naturally occurring and experimental bacterial infections in animals. As discussed below, the reported outcomes of those studies varied dramatically, depending on the infectious agents used, the animal models of infection, and the lytic potency of bacteriophages.

13.2.2. Early Studies Examining the Efficacy of Bacteriophages for Preventing and Treating Animal Infections

One of the earliest animal models used in several phage therapy studies was murine salmonellosis—a systemic disease produced in susceptible mice by several serotypes of *Salmonella*, including typhimurium, enteritidis, dublin, choleraesuis, and abortus. For example, Topley et al. (1925) used an *S.* Typhimurium strain to evaluate the efficacy of phage treatment in experimentally infected mice. After bacterial challenge, mice were administered phage *per os* and the mortality rates of, and bacterial shedding from, phage-untreated and phage-treated mice were analyzed. In contrast to d'Herelle's observations with *Salmonella*-infected chickens, the authors found that phage administration did not reduce mortality or bacterial shedding. Furthermore, a study by Topley, et al. (1925) revealed that intraperitoneal (i.p.) injection of their phages did not significantly reduce the spread of the *S.* Typhimurium infection among the mice. Several factors may have contributed to the failure of phage treatment in these two studies, including the use of phages that were not optimally effective against the challenge strain of *S.* Typhimurium; indeed, the phage apparently failed to completely lyse that strain *in vitro* (Topley and Wilson, 1925). In that context, Fisk (1938) reported that injecting antityphoid phages with good *in vitro* activity against the challenge strain into mice before challenge with typhoid bacilli conferred excellent protection. The phage-inoculated mice were protected for 24 h; phage therapy initiated 4 h after bacterial challenge also protected mice. Protection was not observed when the phage were heat-inactivated (70°C, 50 min), thus indicating that viable phage were required for the preparation's efficacy.

In some early studies (Arnold and Weiss, 1926; Asheshov et al., 1937), authors simultaneously administered bacteria and phage to animals—an approach which may not be relevant to most real-life situations and may yield potentially misleading results. In this context, bacteria-phage interactions usually occur very rapidly; e.g., although bacterial lysis by phages may take 20–40 minutes, phage attachment to the bacterial membrane (followed by phage DNA injection) may occur within just a few seconds or minutes (see Chapters 3 and 7). Thus, mixing the bacteria with the phage before inoculating the animals with the mixture can yield results that primarily represent *in vitro* interaction of the bacteria in the inoculum, rather than results pertaining to the efficacy of phages in preventing or treating bacterial infections *in vivo*.

Early attempts at phage treatment of experimentally induced staphylococcal and streptococcal septicaemias in rabbits and mice were reported to be unsuccessful by several investigators (Clark and Clark, 1927; Krueger et al., 1932), including Giorgi Eliava (1930)—the co-founder of the Bacteriophage Institute in Tbilisi, Georgia (see Chapter 14). Also, many attempts to treat experimental plague in rabbits, guinea pigs, rats, and mice failed to influence the course of the disease (Naidu and Avari, 1932; Compton, 1930; 1928; Colvin, 1932). Moreover, in clear contrast to d'Herelle's earlier studies, Pyle (1926) reported phage therapy to be ineffective in treating fowl typhoid, even though he used phage with excellent *in vitro* activity against the infecting bacterium. On the other hand, injecting bacteriophages into the carotid artery has been claimed to significantly reduce the mortality of rabbits with experimental streptococcal meningitis (Kolmer and Rule, 1933). Also, *E. coli* cystitis in rabbits and guinea pigs has been reported (Marcuse, 1924; Larkum, 1926) to be cured or markedly alleviated by phage treatment. Furthermore, excellent results were reported by Dubos et al. (1943) who used intraperitoneal phage to treat cerebrally injected *S. dysenteriae* infections in mice. (These studies are discussed in some detail in Chapter 14.)

13.2.3. Recent Studies Examining the Efficacy of Bacteriophages for Preventing and Treating Animal Infections

13.2.3.1. *Salmonella* Infections

After the early work by d'Herelle (1921; 1926) and other investigators (1925; Topley and Wilson 1925; Fisk 1938), *Salmonella*-infected laboratory animals re-emerged as one of the most commonly used *in vivo* models to study the prophylactic and therapeutic value of bacteriophages. For example Berchieri, *et al.* (1991) isolated several bacteriophages lytic for *Salmonella* Typhimurium from chickens, chicken feed, and human sewage systems in England and evaluated their efficacy in treating experimental *S.* Typhimurium infections in chickens. The challenge strain produced a fatal infection in ca. 53% of the phage-untreated chickens, and administration of some of their phages produced a statistically significant reduction in chicken mortality. One of the most effective phages, ϕ2.2, also significantly reduced (i) the mortality caused by two other virulent strains of *S.* Typhimurium and (ii) viable numbers of the challenge strain in the alimentary tracts of chickens treated with the phage. For example, a 1 log reduction was observed in the number of challenge bacteria in the crop, small intestines and caeca at 12 h postchallenge and an 0.9 log reduction was seen in the liver at 24 and 48 h post-challenge. The authors hypothesized that, because of the challenge strain's relatively low LD_{50} dose of 10^3 CFU, that level of reduction might be clinically significant. A few phage-resistant colonies were isolated from chickens' caecal contents; however, they were of the rough (i.e., avirulent or less-virulent) phenotype. Also, no phage-neutralizing antibodies were detected in serum obtained from chickens sacrificed 32 days post challenge. The results also indicated that the inoculated phage, which was highly lytic *in vitro*, only persisted in the intestine as long as the *Salmonella* count remained high.

In all cases, administration of high-titer phage preparations was required for a positive therapeutic effect. The timing of the phage treatment was also important; i.e., initiating phage treatment shortly after the bacterial challenge was significantly more effective than was delaying the treatment. At the present time, the mechanism(s) for this time-dependence are not clear. However, since *Salmonella* is an intracellular pathogen, one possible explanation is that early administration of phages kills most of the salmonellae in the gut before they are internalized and, thus, protected from the lytic effect of phages. More research is likely to provide much needed information in that regard, and to generate critical data needed for the optimal design and implementation of phage-mediated prophylaxis and therapy of *Salmonella* infections in various agriculturally important animals.

13.2.3.2. *Escherichia coli* Infections

13.2.3.2.1. Smith and Huggins' Studies

E. coli can cause several types of noninvasive enteritis and septicemia in various animal species. Thus, initial studies examining the possible therapeutic value of phage in animals focused on *E. coli* infections, such as Larkum's 1926 work on phage therapy for *E. coli* cystitis in rabbits and guinea pigs (Larkum, 1926). After a long period of disinterest in phage therapy (see Chapter 14), *E. coli*-infected animal models once again emerged as a primary system to evaluate the possible efficacy of phage. Arguably the best-known studies of phage therapy in veterinary medicine were reported from the laboratory of Herbert Williams Smith and his colleagues at the Institute for Animal Disease Research in Houghton, Cambridgeshire, Great Britain. Their early work focused on *E. coli* septicemia experimentally induced in mice using a strain of *E. coli* O18:K1:H7 ColV$^+$ from a child with meningitis (Smith and Huggins, 1982). Some of the phages used during the study were specific for the capsular K1 antigen—a major virulence determinant in the challenge *E. coli* strain. A single intramuscular (i.m.) injection of one anti-K1 phage was more effective than were multiple i.m. injections of various antibiotics (tetracycline, ampicillin, chloramphenicol, or trimethoprim plus sulphafurazole) in protecting mice against a potentially lethal, i.m.- or intracerebrally (i.c.)-induced infection with the challenge *E. coli* strain. The phage treatment also was reported to be at least as effective as were multiple i.m. injections of streptomycin. Experiments enumerating the phages and bacterial pathogen in various animal tissues revealed that the phages persisted for

24 hours in the bloodstream, and that high phage titers (10^6 PFU/gram of tissue) persisted for several days in the spleen. A few phage-resistant mutants were identified during the study; however, they had the $K1^-$ phenotype, and thus were significantly less virulent than the wild-type $K1^+$ strain. Phage administration 3 to 5 days before the *E. coli* challenge also protected mice against the experimentally induced infection, although the protective effect varied among phages propagated on different bacterial host strains. The excellent results obtained during their initial study prompted the authors to expand their research to other animals.

During their second study, Smith and Huggins (1983) examined the therapeutic and prophylactic efficacy of *E. coli*-specific bacteriophage preparations in neonatal enteritis in calves, piglets, and lambs. During the experiments in calves, 71 newborn calves were infected orally with ca. 3×10^9 CFU of an O9:K30.99 enterotoxigenic strain of *E. coli*. Fifty-seven of the seventy-one calves were colostrum-fed; they were susceptible to diarrhea but resistant to septicemia. The calves were divided into 6 groups, 4 of which were treated with a mixture of two different *E. coli*-specific phages (Table 13.1). None of the nine calves treated with 10^{11} PFU of a phage mixture 8 h after bacterial challenge became ill. In addition, only 2 animals died in a group of 13 colostrum-deprived calves who received phages at the onset of diarrhea. In contrast, the mortality rate in the phage-untreated control groups was 93% among the colostrum-treated calves and 100% among the colostrum-free calves (Table 13.1). If administration of the phage treatment was delayed until the onset of diarrhea, the disease was not prevented; however, the severity of the illness was significantly reduced and so was the mortality. The phage did not totally eliminate the pathogenic *E. coli* strain from the gut, but appeared to reduce their numbers to a level which was below that required to produce disease. The phage persisted in the gut as long as their host strain's numbers remained high, and disappeared thereafter. Similar treatments with other specific phage preparations were effective in protecting piglets and lambs against challenge with enterotoxigenic strains of *E. coli* O20:K101.987P and *E. coli* O8:K85.99, respectively, and no phage-resistant mutants were identified in the phage-treated lambs. A few phage-resistant *E. coli* mutants were seen in the phage-treated calves and piglets; however, they had the $K30^-$ or $K101^-$ phenotype and, when tested in animals, appeared to have significantly lower virulence than the parental, wild-type strains.

In a subsequent series of studies, Smith et al. (1987b; 1987a) again used *E. coli*-infected calves to evaluate further the efficacy of phage treatment. During one of these studies (Smith et al., 1987b), they also evaluated the susceptibility of phages to various conditions likely to be encountered after their administration into animals. The authors noted that the low pH of the abomasum's contents affected the viability of orally administered phages, but that the deleterious effect was reduced if phages were administered shortly after milk feed. A similar effect was achieved by sodium bicarbonate administration in the feed just prior to or during oral phage treatment. In this context, it is noteworthy that neutralizing gastric acid by oral administration of bicarbonate mineral water shortly before phage administration is a common practice during human phage therapy studies in the former Soviet Union and Eastern Europe (see Chapter 14).

TABLE 13.1
Data from the 1983 Study by Smith and Huggins (1983). The Study Examined the Efficacy of Phage Therapy in Experimental *E. coli* Diarrhea in Calves, Piglets and Lambs

Animals/No of Animals	Colostrum Fed	Phage or Phage Mixture	Time Phage Given (after bacterial challenge)	Animals with Diarrhea	Dead Animals	Mortality
Calves		1×10^{11} PFU/dose				
6	No	B44/1 & B44/2	1 h	3	1	17%
8	No	No Phage	N/A	8	8	100%
9	Yes	B44/1 & B44/2	8 h	0	0	0%
21	Yes	B44/1 & B44/2	At onset of diarrhea	21	14	67%
13	Yes	B44/1 & B44/3	At onset of diarrhea	13	2	15%
14	Yes	No Phage	N/A	13	13	93%
Piglets		1×10^{10} PFU/dose				
7	N/A	P433/1 & P433/2	At onset of diarrhea		0	0%
7	N/A	No Phage	N/A		4	57%
Lambs[a]		1×10^9 PFU/dose				
4	N/A	S13	8 h	2	0	0%
4	N/A	No Phage	N/A	4	1	25%
		1×10^{10} PFU/dose				
3	N/A	S13	8 h	2	0	0%
3	N/A	No Phage	N/A	3	1	25%

N/A—Not applicable

[a]The study was terminated at 24 h post bacterial challenge, at which time all remaining animals were sacrificed, and the levels of the challenge strain in their intestines was evaluated. The mortality for this group is based on the number of animal that died from infection under 24 h.

13.2.3.2.2. *Experimental* E. coli *Infections in Chickens and Colostrum-Deprived Calves*

The studies by Smith and Huggins, which have been reviewed by several authors (Barrow and Soothill, 1997; Sulakvelidze et al., 2001) and examined using mathematical models and statistical analyses (Levin and Bull, 1996), have stimulated the recent rekindling of interest in phage therapy in the West. For example, Barrow et al. (1998) used an *E. coli*-specific bacteriophage (previously isolated from sewage and found to attach to the K1 capsular antigen) to prevent septicemia and a meningitis-like infection in chickens caused by a $K1^+$ strain of *E. coli*. In untreated chickens, the experimental infection had a mortality rate of almost 100% after i.m inoculation with 10^6 CFU; however, a single i.m. injection of the phage preparation, prior to bacterial challenge, prevented morbidity and death. The phage treatment's efficacy was dose-dependent: the best protection was obtained when high doses (e.g., 10^6 PFU) of phages were injected. The injection of 10^4 PFU provided significant, albeit less, protection, and 10^2 PFU did not significantly protect the chickens. The protection also was obtained when phage administration was delayed until the signs of disease were evident.

Similarly encouraging results were obtained in the experiments with calves. For example, orally challenging (ca. 10^{10} CFU, by stomach tube) two colostrum-deprived calves with the $K1^+$ strain of *E. coli* elicited severe septicemic disease within 18–36 h post-challenge, at which time they were sacrificed for humane reasons. In contrast, three of four calves injected i.m. with the phage preparation (10^{10} PFU) 8 h post-challenge remained healthy, and the fourth calf appeared to be only slightly ill (Barrow et al., 1998). The number of calves examined during the study was too small for statistical analysis, but the phage treatment did appear to have had a positive therapeutic effect.

13.2.3.2.3. E. coli *Infections in Mice and "Long-Circulating" Bacteriophages*

An *E. coli*-infected mouse model recently was used to evaluate the *in vivo* efficacy of "long-circulating" bacteriophages as antimicrobial agents (Merril et al., 1996). The authors hypothesized that phage therapy may be deleteriously affected by the rapid elimination of phages by various mammalian host defense mechanisms, particularly by the reticuloendothelial system. Thus, in order to reduce phage elimination, they used a natural selection strategy (which they called the "serial passage" method) to obtain *E. coli* phages having an increased ability to remain in the bloodstream of mice. For example, to isolate λ phage mutants possessing that capability, mice were injected i.p. with 10^{11} PFU of λ phage W60, blood samples were obtained 7 h post-injection, the residual phages present in bloodstream were injected into mice, and the serial cycling of the phages (injection into animals, isolation, and regrowth) was repeated nine more times. Using this approach, the authors isolated *E. coli* phage λ W60 mutants and *S.* Typhimurium phage P22 mutants capable of staying in the circulation of mice for significantly longer than did the wild-type, parental phages. After their successful mutant isolation experiments, the authors compared the relative abilities of the wild-type λ phage and the "serially passaged" *E. coli* phage mutants to protect 1-week-old BALB/c mice against an experimental *E. coli* septicemia, and they found that a single i.p. injection of either of the two long circulating phages was more effective in rescuing bacteremic mice than was a single injection of the wild-type phage, which suggests that the persistence of high phage titers in the circulation has a positive impact on the efficacy of phage treatment (see Chapter 14 for more details about the study).

13.2.3.2.4. E. coli *Respiratory Infections in Chickens*

The ability of phage therapy to prevent fatal *E. coli* respiratory infections in broiler chickens was recently evaluated in a series of studies at the University of Arkansas. During their first study (Huff et al., 2002b), the authors performed three separate experiments. The first experiment involved challenging (air sac inoculation) groups of 3-day-old-chickens with mixtures containing (i) 10^3 CFU of *E. coli* and 10^3 or 10^6 PFU of the phages, and (ii) 10^4 CFU of *E. coli* and 10^4 or 10^8 PFU of the phages. In the second experiment, 1-week-old birds drank water containing 10^3 or 10^4 PFU of the phages/ml before being air sac-challenged with 10^3 CFU of *E. coli*, or they ingested water containing 10^4 or 10^6 PFU of phages/ml before challenge with 10^4 CFU of *E. coli*. During the third experiment, 1-week-old chickens were air sac-challenged with 10^4 CFU of *E. coli*, and they then drank water containing 10^5 or 10^6 PFU of phages/ml. In the first experiment, the chickens challenged with 10^3 CFU of *E. coli* exhibited a mortality rate of 80%; however, the mortality rate decreased to 25% and 5% when the birds were challenged with a mixture containing the bacteria and 10^3 or 10^6 PFU of phages, respectively. In addition, chickens challenged with 10^4 CFU of *E. coli* had a mortality rate of 85%, which decreased to 35% and 0% when they were challenged with a mixture containing the bacteria and 10^4 or 10^8 PFU of phages, respectively. On the other hand, phage administration did not protect the chickens in the second and third experiments. In their second study (Huff et al., 2002a), the authors reported that aerosol administration of bacteriophages was efficacious in preventing fatal *E. coli* respiratory infections in broiler chickens. The authors' third study (Huff et al., 2003b) compared the efficacy of aerosol administration and i.m. injection of bacteriophages in preventing fatal *E. coli* respiratory infections in broiler chickens. Their most recent study examined the efficacy of multiple vs. single i.m. injections of bacteriophage to treat a severe *E. coli* respiratory infection in broiler chickens (Huff et al., 2003a). As in their previous studies, the authors found that bacteriophages protected against a fatal respiratory challenge with *E. coli*, and that the outcome of the phage treatment was dependent on, among other factors, the route of phage administration (e.g., adding the bacteriophages to the drinking water did not protect the birds). Although the results of the above-described studies appear to be encouraging, they should be interpreted cautiously. For example, as described in section 13.2.2 of this Chapter, mixing phage with bacteria may result in phage infecting the bacteria prior to inoculation rather than *in vivo*. Thus, some or all of the reported positive results might be artifacts caused by the experimental protocols.

13.2.3.3. *Enterococcus faecium* Infections

Recently, the value of using bacteriophages for preventing or treating bacteremia caused by vancomycin-resistant enterococci (VRE) has been evaluated in an experimentally infected mouse model (Biswas et al., 2002). The authors developed an

E. faecium-infected mouse model to determine the efficacy of phage treatment in preventing a fatal septicemia elicited by i.p. injection of 10^9 CFU of a vancomycin-resistant *E. faecium* strain. A single i.p. injection (administered 45 min after the bacterial challenge) of 3×10^9 or 3×10^8 PFU of a phage preparation with potent *in vitro* lytic activity against the bacterium protected all of the infected mice; the efficacy of treatment was clearly dose-dependent (Fig. 13.1). When treatment was delayed until the animals were moribund (18 to 24 h postchallenge), ca. 50% of them still were rescued by a single injection of the phage preparation. In both instances, survival was associated with a significant decrease in the number of challenge bacteria in the animal's bloodstream.

They also explored whether their observed protective effect required viable phages that could grow in the bacterial host, or whether "phage rescue" might have been a function of non-specific immune response triggered by the injection of the phage preparation. A phage preparation (ca. 1×10^{10} PFU/ml) was heat-treated (80°C, 20 min) and the destruction of viable phages confirmed by plaque assays. Four days after challenge with *E. faecium*, 80% of the mice treated with the viable, plaque-forming phage preparation survived; however, only 10% of the mice injected with the heat-inactivated phage preparation survived. The latter percentage was identical to that of the mice in the control group (i.e., mice treated with PBS). The results were statistically significant ($P < 0.0006$). The authors also addressed the possibility that the observed effect of heat-treatment may have resulted from inactivation or denaturation of other components (e.g., media components) of phage preparation that might have possessed therapeutic, immunostimulatory activity by comparing the *in vivo* protective abilities of two *E. faecium* phages with different *in vitro* lytic activities against the challenge strain of *E. faecium* (i.e., one phage formed plaques, and one strain did not form plaques, when grown on a lawn of the challenge strain). All of the infected mice treated with the lytic phage preparation survived, compared with survival rates of 20% and 50% for infected mice treated with the nonlytic phage preparation and with phosphate buffered saline (PBS) buffer, respectively ($P < 0.03$). This finding further supports the idea that the observed therapeutic effect in mice was due to the presence of viable phages with lytic activity against the challenge strain, rather than by specific or nonspecific immunostimulation. (The therapeutic mode of action of bacteriophages is discussed further in section 13.4 of this chapter and section 14.5 of Chapter 14.) The multiply injected mice did not exhibit anaphylactic reactions, fever, or any other side effects.

13.2.3.4. *Vibrio cholerae* Infections

Sarkar et al. (1996) examined the ability of *V. cholerae*-typing phages to reduce *in vivo* levels of *V. cholerae*, and to reduce fluid accumulation caused by cholera toxin, in the rabbit ileal loop (RIL) model. Ten phages (ATCC 51352 B1 to B10; $10^{10} - 10^{11}$ PFU/ml of each preparation) were injected, alone or together with 10^8 CFU of the challenge *V. cholerae* strain (MAK 757 or ATCC 51352), into ileal loop segments of outbred New Zealand rabbits. The PBS buffer was used as the negative control, and the toxigenic strain *V. cholerae* 569B Inaba was used as a positive control. The animals were sacrificed ca. 18 h post-treatment, and the fluid accumulation ratios

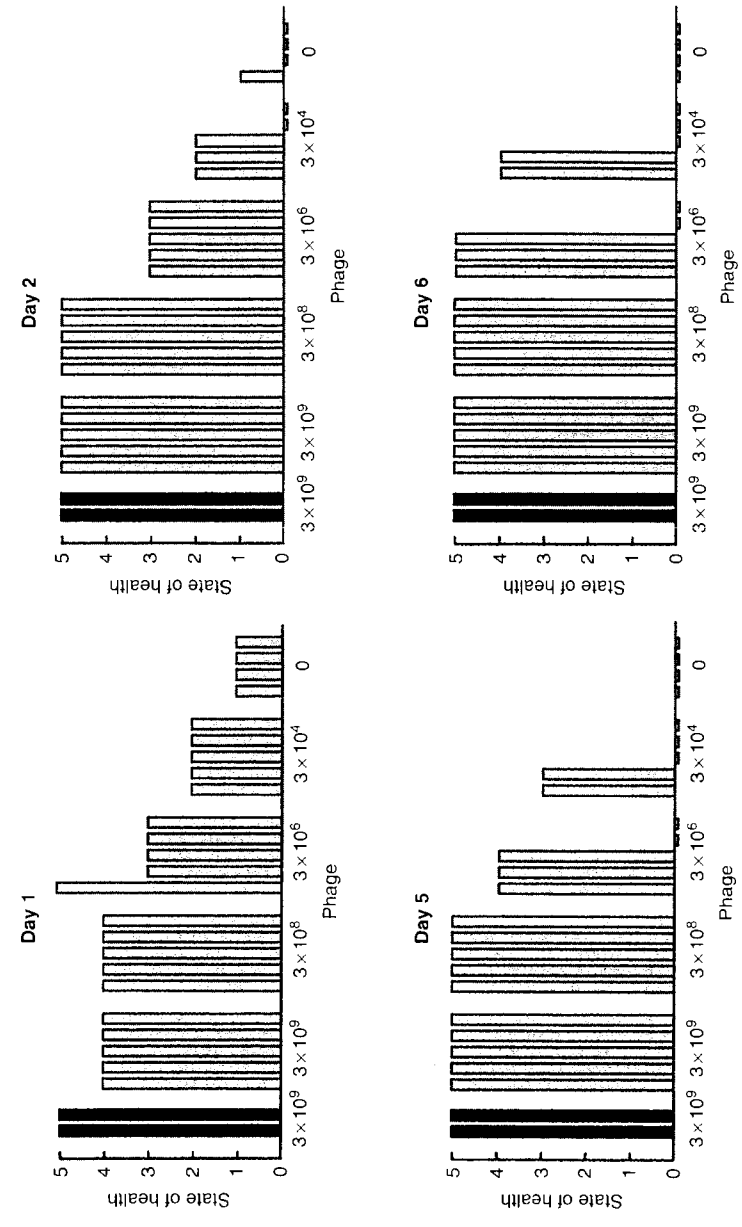

FIGURE 13.1 Efficacy and dose effect of an *E. faecium*-specific phage in protecting mice from a lethal VRE septicemia. The group on the far right (four mice) was an untreated control, which was injected i.p. with phage-free buffer instead of the phage preparation. The group on the far left (two animals) was a phage control, which was injected with the phage preparation and was not infected with *E. faecium* (from Biswas et al., 2002, reprinted with permission from the American Society for Microbiology, Washington, D.C., U.S.A.).

and the number of challenge bacteria in the RIL were determined. Phage administration was reported not to have a prophylactic effect, since it (i) did not reduce the number of challenge bacteria in the RIL, and (ii) did not reduce the fluid accumulation ratios in the RIL. The study did not specifically examine the *in vitro* ability of the phages to lyse the challenge bacteria; however, since a large collection of typing phages was used, it is likely that at least some of them would have had *in vitro* lytic activity against the challenge strain. The authors proposed that, although the anti-*V. cholerae* phages persisted in the RIL throughout the experiments, some component(s) of the intestinal milieu might have inhibited their activity against the challenge *V. cholerae* strain. Given the early history of the extensive and successful use of bacteriophages to prevent and treat human cholera (see Chapter 14 for more information on this subject), the negative outcome of the above study suggests that the RIL model may not be an optimal model for evaluating the efficacy of phages in preventing and treating naturally occurring cholera. It also highlights how little is known concerning the interaction of phages and their targeted bacteria in the mammalian intestinal tract.

13.2.3.5. *Clostridium difficile* Infections

Ramesh et al. (1999) recently characterized the ability of bacteriophages to prevent a fatal, *C. difficile* ileocecitis in experimentally infected hamsters. Their experimental protocols were designed to study many of the factors that influence the efficacy of phage therapy in animals; e.g., the persistence of phages in the mammalian body, the effect of gastric acidity on phage viability, optimal dosing regimens, etc. The bacteriophage used in the study was isolated from a lysogenic strain of *C. difficile*, and the experimental animals used during the study consisted of 26 adult hamsters. Groups 1a, 1b and 1c each contained 6 hamsters and groups 2 and 3 each contained 4 hamsters. In order to facilitate the *C. difficile* ileocecitis, the hamsters in groups 1 and 2 were pretreated with clindamycin (3 mg/100 g body weight, administered intragastrically); the hamsters in control group 3 received saline instead of the antibiotic. Twenty-four hours after the clindamycin-pretreatment step, the gastric acidity of the hamsters in all three groups was neutralized (via orogastric administration of 1 M bicarbonate buffer; 1 ml/animal), and the animals were intragastrically inoculated with a suspension (1 ml containing 10^3 CFU) of the *C. difficile* challenge strain. Immediately after the bacterial challenge step, the hamsters in subgroups 1a, 1b, and 1c were treated with the phage preparation (1 ml containing 10^8 PFU). The animals in subgroups 1b and 1c also received additional doses of the phage preparation, at 8 h intervals, for 48 h and 72 h, respectively (gastric acidity was neutralized with intragastric administration of bicarbonate buffer immediately before each phage treatment). The hamsters in groups 2 and 3 served as controls; i.e., they were not treated with the bacteriophage preparation. All of the animals in the control groups died within 96 hours after bacterial challenge; however, with the exception of one hamster, all of the phage-treated animals survived. The phages were not detectable in the animals' cecal contents shortly after they received the last dose of phage preparation. In addition, the phage therapy did not have a long-lasting protective effect in the hamsters; i.e., when the surviving hamsters were pre-treated with clindamycin and rechallenged with *C. difficile* (2 weeks after stopping phage therapy), they died within 96 hours postchallenge. The *C. difficile* phage used during the study has not been carefully characterized, and it may not have been an optimal choice for phage therapy because of its lysogenic potential and, thus, its possible less-than-optimal lytic activity. Nevertheless, the study's results indicate that *C. difficile* infections are amenable to phage therapy, and they highlight the importance of neutralizing gastric acidity prior to phage treatment via the oral route. As noted earlier, a gastric acidity neutralization protocol also has been suggested by other investigators (Smith et al., 1987b), and it was common practice during human phage therapy studies in the former Soviet Union and Eastern Europe (see Chapter 14).

13.2.3.6. *Acinetobacter baumanii, Pseudomonas aeruginosa,* and *Staphylococcus aureus* Infections

Soothill (1992) examined the efficacy of phages in treating experimental murine infections caused by *A. baumanii*, *P. aeruginosa*, and *S. aureus*. The *A. baumanii*- and *P. aeruginosa*-specific phages were isolated from sewage in Birmingham, United Kingdom, and the *S. aureus*-specific phage ϕ-131 was obtained from the Hirszfeld Institute of Immunology and Experimental Therapy in Wroclaw, Poland (see Chapter 14). The protective effects of phage were evaluated in groups of adult outbred mice injected i.p. with a predetermined lethal dose of bacteria inoculated simultaneously with the phage preparations. A decrease in body temperature (to 34°C) was found to be an accurate predictor of terminal illness; therefore, when the body temperature of the infected mice reached 34°C, they were sacrificed and their organs and tissues were analyzed for the presence of the challenge bacteria and bacteriophages. As few as 10^2 PFU of the *A. baumanii*-specific phage protected mice against 5 LD_{50} doses of a virulent strain of *A. baumanii*, and the phages multiplied in the mice during the course of the experiment. A positive outcome was also associated with negative *A. baumanii* cultures from the phage-treated mice. A much higher dose of the anti-*P. aeruginosa* phage preparation was required to protect mice against *P. aeruginosa* challenge, but the protective effect was significant against 5 and 10 LD_{50} doses of the bacterium (Table 13.2). In contrast, the *S. aureus*-specific phage (which was poorly lytic *in vitro*) did not protect mice from the lethal effects of the two challenge strains of *S. aureus*. Interestingly, the same *S. aureus* phage has been used extensively and successfully to treat human *S. aureus* infections in Poland (Slopek et al., 1987). The reason for the seeming discrepancy in results is not clear. Since one of Soothill's challenge strains was a clinical isolate obtained from Poland (where it also was used as a host strain for ϕ-131), host-strain differences do not explain the different results. The number of animals used during Soothill's studies was small, complicating rigorous statistical analysis of his data. Also, the phage preparations were injected simultaneously and by the same route as were the bacteria, and into a body cavity which gave a good opportunity for the phages to attach to the target bacteria; i.e., conditions which are not likely to be encountered in real-life settings involving phage therapy of established *A. baumanii* and *P. aeruginosa* infections. Thus, although Soothill's results are promising, they require confirmation in a more clinically relevant animal model. Interestingly, the *P. aeruginosa*-specific phage (BS24) used

TABLE 13.2
Data from the 1992 Study of Soothill (Soothill, 1992). The Study Examined the Value of Phage Therapy in Experimental Murine Infections Caused by *A. baumanii, P. aeruginosa* and *S. aureus*[a]

Phage Dose	Mortality	Phage Dose	Mortality	Phage Dose	Mortality
A. baumanii 1.5×10^8 CFU		*A. baumanii* 9.5×10^7 CFU		*A. baumanii* 5.6×10^7 CFU	
(Challenge with $8 \times LD_{50}$)		(Challenge with $5 \times LD_{50}$)		(Challenge with $3 \times LD_{50}$)	
Two mice per dose		One mouse per dose		Five mice per dose	
8.3×10^6 PFU	0%	1.0×10^2 PFU	20%	1.2×10^3 PFU	0%
1.7×10^6 PFU	0%	3.6×10^1 PFU	100%	1.2×10^2 PFU	0%
3.3×10^5 PFU	0%	1.2×10^1 PFU	100%	1.2×10^1 PFU	0%
6.6×10^4 PFU	0%	4 PFU	100%	1 PFU	100%
1.3×10^4 PFU	0%	No Phage	100%	No Phage	100%
No Phage	100%				
P. aeruginosa 1.5×10^8 CFU		*P. aeruginosa* 8.0×10^7 CFU			
(Challenge with $10 \times LD_{50}$)		(Challenge with $5 \times LD_{50}$)			
Five mice per dose		Five mice per dose			
2.9×10^8 PFU	0%	1.8×10^7 PFU	20%		
2.9×10^7 PFU	80%	6.0×10^6 PFU	100%		
5.8×10^6 PFU	100%	2.0×10^6 PFU	80%		
2.9×10^5 PFU	100%	6.7×10^5 PFU	100%		
No Phage	100%	No Phage	100%		

[a]The *S. aureus*-specific phage (ϕ-131) was poorly lytic *in vitro*, and it did not protect mice against *S. aureus*; thus, *S. aureus*-related data are not included in the table.

by Soothill has been later reported to prevent *P. aeruginosa*-mediated destruction of pigskin *in vitro* (Soothill et al., 1988) and to protect against *P. aeruginosa*-associated destruction of skin grafts *in vivo* (Soothill, 1994). The successful outcome of the latter study suggests that the local application of phage might be a useful tool for preventing and treating *P. aeruginosa* infections in burn grafts.

13.2.4. Bacteriophages As a Potential Tool for Reducing Antibiotic Usage in Agribusiness

An estimated 50% to 70% of the antibiotics used in the United States are given to farm animals (Gustafson, 1991), for three main purposes: (i) prophylactically, to prevent disease in flocks and herds, (ii) to treat sick livestock (the antibiotics used to treat the animals are usually the same as those used in human medicine, and they are administrated via drinking water or feed over a period of several days), and (iii) to improve digestion and utilization of feed, which often results in improved weight gain. Antibiotics used in the latter setting often are referred to as *growth-promoting antibiotics* or GPAs. Most GPAs are not commonly used in human medicine, and they are usually administered, in small amounts, to poultry and other livestock via feed. The use of antibiotics in livestock has become a major source of concern among public health officials, consumer groups, and livestock industry leaders because of the possibility that they contribute to the declining efficacy of antibiotics used to treat bacterial infections in humans (Smith et al., 2002). This concern caused the European Union to ban the use of four antibiotics (virginiamycin, bacitracin zinc, spiramycin, and tylosin phosphate) as additives in animal feeds, and a complete ban on all GPAs is likely to take effect in Europe in 2006 (Ferber 2003). Although no similar ban has yet been introduced in the United States, the US Food and Drug Administration has recently proposed regulations that could impose severe limitations on the future agricultural and farm-veterinary use of all antibiotics. Moreover, bills that would curb the use of animal antibiotics that are similar to human antibiotics have already been introduced in the U.S. Senate and House of Representatives (Ferber, 2003). Banning or markedly reducing the agricultural and farm-veterinary use of antibiotics may have a negative impact on the safety of foods and on the treatment of sick flocks or herds of domesticated livestock—unless effective, safe, and environmentally friendly alternatives can be developed. Bacteriophage-based antibacterial products may be one such alternative.

Phages are ubiquitous in the environment and their use in livestock is likely to provide one of the most environmentally friendly antibacterial approaches available today. In addition, phages' several important advantages over antibiotics (Pirisi, 2000) make their use in various livestock industries potentially very appealing. For example:

- Because of the specificity of phages, their use in agriculture is not likely to select for phage resistance in untargeted bacterial species; whereas, because of their broad spectrum of activity, antibiotics select for many resistant bacterial species, not just for resistant mutants of the targeted bacteria.
- Because the bacterial resistance mechanisms against phages and antibiotics differ, the possible emergence of resistance against phages will not affect the susceptibility of the bacteria to antibiotics used to treat humans—which is the key concern regarding the use of antibiotics in agribusiness.
- Unlike antibiotics (which have a long and expensive development cycle), phage preparations can readily be modified in response to changes in bacterial pathogen populations or susceptibility, and effective therapeutic phage preparations can be rapidly developed against emerging antibiotic- or phage-resistant bacterial mutants.

The range of bacterial pathogens that could be targeted with phages is quite wide, but initial work in this area is likely to start with bacterial pathogens known to be most problematic in poultry and other livestock industries. For example, since necrotic enteritis elicited by *Clostridium perfringens* is a significant cause of morbidity and mortality in chickens, initial studies to reduce the prophylactic and therapeutic use of antibiotics in the poultry industry may focus on determining the value of using phages against that pathogen. Phage preparations could also be developed against other bacterial pathogens that may be of concern for other domesticated livestock;

e.g., phage preparations for treating bovine mastitis should have a substantive practical applicability in the dairy industry. Such preparations (primarily targeting *S. aureus* strains) have been developed in the former Soviet Union, with preliminary results said to be encouraging (T. Gabisonia, personal communication). If initial studies in that direction generate similarly encouraging results in the United States and Western Europe, the approach may lead to the development of several phage-based therapeutic preparations for veterinary medicine. Further bacteriophage research related to agricultural applications could initially focus in two main directions: (i) developing phage preparations to be used as prophylactic or therapeutic antimicrobials (i.e., to directly lyse the targeted pathogens), and (ii) developing phage lysate-based preparations to be used as vaccines. The use of such preparations may help reduce the prophylactic and therapeutic use of antibiotics in farm animals. In addition, it may potentially have some growth-promoting effect in animals (e.g., by reducing stress caused by bacterial infections)—which may help reduce or eliminate the use of GPAs in various livestock industries.

Bacteriophages may also be of value in reducing the use of antibiotics in the aquaculture and farming industries. For example, fire blight, caused by the bacterium *Erwinia amylovora*, is a devastating disease of apple and pear trees in many countries around the world—and many commercial producers rely heavily on antibiotics (e.g., streptomycin) to prevent the disease by reducing the accumulation of epiphytic populations of *E. amylovora* on nutrient-rich stigmatic surfaces of blossoms (Schnabel and Jones, 2001). However, that practice has resulted in the emergence of streptomycin-resistant mutants of *E. amylovora* (Jones and Schnabel, 2000)—a good illustration of how the use of antibiotics in agribusiness may contribute to the emergence of antibiotic-resistant bacterial strains. Although they do not pose a direct health safety problem for humans, the spread of streptomycin-resistant *E. amylovora* strains may contribute to an increased spread of streptomycin-resistance genes (and, possibly, of other antibiotic-resistant genes) among various bacterial species, including those highly pathogenic for humans.

13.3. BACTERIOPHAGES AND DISEASES OF AQUACULTURE AND PLANTS

13.3.1. Plant Infections

Bacteriophages were first proposed as potential agents for controlling bacterial diseases of plants as early as 1926 (Moore, 1926; Okabe and Goto, 1963), and they were successfully used to control Stewart's Disease in corn by Thomas (1935). Phages were used by Civerolo et al., (1969) and by Civerolo (1973) to reduce *Xanthomonas oryzae* Bacterial Spot disease of peach seedlings by 86% to 100%. However, most of the published research concerning phages specific to bacterial plant pathogens has not been treatment-oriented; phages were often simply used as a tool for typing plant-infecting bacteria. Reviews about bacteriophages of various plant pathogens were published by Okabe and Goto (1963) and by Gill and Abedon (2003).

E. amylovora has been one of the most commonly studied plant bacterial pathogens, and several publications in the 1960s described the isolation, characterization, and use of various phages for typing *E. amylovora* strains. The first report in which the possible role of bacteriophages in the epidemiology of *E. amylovora*-associated fire blight was discussed came from Erskine (1973). Subsequently, *E. amylovora*-specific phage ϕEa1 was successfully used to treat/prevent fire blight in apple seedlings inoculated with *E. amylovora* (Ritchie and Klos, 1979; 1977). Treatment with the polysaccharide depolymerase encoded by ϕEa1 has also been reported to attenuate the symptoms of fire blight in pear fruit inoculated with *E. amylovora* (Hartung et al., 1988). Schnabel et al. (2001) recently reported isolating several *E. amylovora*-specific bacteriophages (some identical or closely related to ϕEa1) from various fruits and soil samples collected at sites displaying fire blight symptoms. More recently, Gill et al. (2003) reported isolating more than 40 *E. amylovora*-specific bacteriophages from sites in and around the Niagara region of southern Ontario and the Royal Botanical Gardens in Hamilton, Ontario. Molecular characterization of the phages with Polymerase Chain Reaction (PCR) and Restriction Fragment Length Polymorphism (RFLP) revealed that some of them were closely related to ϕEa1. A study of the host ranges of the phages revealed that certain types were unable to lyse some *E. amylovora* strains efficiently, and that the phages' lytic potential was not limited to that species (i.e., some phages also were capable of lysing the epiphytic bacterium *Pantoea agglomerans*). The authors indicated that investigating the potential of their phages as biocontrol agents would be the subject of their future research.

Such explorations have been conducted by other investigators for other bacterial diseases of plants and for other, perhaps somewhat unusual—but related—applications. In the latter context, colonization with *E. herbicola* and *Pseudomonas syringae* are thought to contribute to the susceptibility of some plant species to tissue damage caused by exposure to low temperatures, by acting as ice-forming nuclei at 1–3°C. Kozloff et al. (1983) received a US patent based on the idea that phages targeting those two species could be used to reduce their population on plant leaves and increase the frost-resistance of phage-treated plants. The results of their experiments suggested that plants infected with *E. herbicola* and treated with *E. herbicola*-specific phages sustained 20%–25% less damage than did plants experimentally challenged with *E. herbicola* but not treated with phages.

Jackson (1989) was granted a US patent for the use of phage preparations for (i) eliminating naturally occurring *P. syringae* from contaminated bean culls, and (ii) reducing the severity of disease symptoms in bean leaves experimentally infected with *P. syringae*. The same author subsequently developed phage preparations targeting *Ralstonia solanacearum* and *Xanthomonas campestris* pv. vesicatoria, the bacterial pathogens responsible for the two main diseases of tomato plants, known as Bacterial Wilt and Bacterial Spot, respectively. In the field trials, phage mixtures were typically poured over the soil at the base of 6- to 8-week-old plants; in the greenhouses, phage were applied in irrigation water (Fox, 2000). Two weeks after inoculation with the pathogen, 60% of the plants without the phage treatment had more than 12% defoliation, while defoliation in all phage-treated plants was less than 12%. The visual results were impressive (Fig. 13.2). The results of these studies are encouraging, and they suggest that bacteriophages may indeed be valuable tools for dealing with various plant diseases of bacterial origin. However, the studies have

FIGURE 13.2 Tomato plants treated with phages 3 and 5 days prior to inoculation with *Ralstonia solanacearum* (center and right plants, respectively), and an inoculated, phage-untreated control plant (left) (from Fox, 2000, reprinted with permission from the American Society for Microbiology, Washington, D.C., U.S.A.).

not yet been published in the peer-reviewed literature, and several critical details pertaining to the studies' design, the characteristics of the phages used, and so forth are not yet available for critical review.

13.3.2. Infections of Aquacultured Fish

Terrestrial animals and plants are not the only candidates for phage therapy, and the possible value of using phages to treat bacterial diseases of aquacultured fish has been gaining increased attention lately. One of the first reports in that area focused on using *Lactococcus garvieae*-specific phages for treating experimentally infected young yellowtail (*Seriola quinqueradiata*) (Nakai et al., 1999). Fish were experimentally infected by i.p. injection of the challenge *L. garvieae* strain, followed by i.p. or oral administration of phages with *in vitro* lytic activity against the challenge strain. All the infected fish that received the phage treatment survived, compared to only 10% of the phage-untreated fish. The strongest protective effect was observed with the fish that were treated with phages at the earliest time after infection with the bacterium. Protection was also observed in yellowtail that received phage-impregnated feed. The authors also analyzed the fishes' internal organs for the presence of phages, and they tested *L. garvieae* isolates recovered from dead fish for phage susceptibility. All of the isolates examined remained susceptible to the phages, and phage-neutralizing antibodies were not detected in serum samples obtained from the yellowtail (Nakai et al., 1999; Nakai and Park, 2002).

Similar studies were subsequently conducted for treating *Pseudomonas plecoglossicida*-caused bacterial hemorrhagic ascites disease in cultured ayu fish (*Plecoglossus altivelis*) (Park et al., 2000; Park and Nakai, 2003). *P. plecoglossicida* is an opportunistic pathogen that can, under certain circumstances, cause disease with a high mortality rate in cultured ayu fish (Nishimori et al., 2000). Phages lytic for *P. plecoglossicida* were isolated from diseased ayu and the rearing pond water obtained from various fish farms in Tokushima Prefecture in Japan. In the first experiment, four groups of 20 ayu (10 g average weight) were fed commercial dry pellets impregnated with a live culture of *P. plecoglossicida* (10^7 CFU/g of pellet). After 15 min of feeding, two of the groups were immediately fed pellets impregnated with a phage suspension (10^7 PFU/g pellet). The control groups received regular feed without phage. The second experiment was designed in essentially the same manner, except that: (i) smaller fish were used (2.4 g average weight), (ii) the sample size was larger; i.e., each group contained 40–50 ayu, and (iii) one test group was treated with phages 1 h after feeding them bacteria-impregnated pellets, and another test group was treated with phages 24 h after feeding with the bacteria-impregnated pellets. The study duration in both cases was two weeks. In the first experiment, fish in the control (i.e., phage-untreated) group begin to die 7 days after the bacterial challenge, and the cumulative mortality rate in that group by the end of the experiment was ca. 65%. In contrast, the mortality rate in the phage-treated group was ca. 23% ($P < 0.001$). Phage treatment also reduced the mortality rate in the phage-treated group in the second experiment; noteworthy, phage administration 1 h after bacterial challenge was significantly more effective in preventing death than was phage administration 24 h after bacterial challenge (0% vs. 13% mortality, respectively). *P. plecoglossicida* was re-isolated from fish kidneys in the first experiment, and all of the cultures were found to be susceptible to the phages used in the study. In addition, phage-resistant mutants selected for *in vitro* appeared to be less virulent for ayu (Park et al., 2000).

The therapeutic efficacy of the anti-*P. plecoglossicida* phages used in these studies was recently further elucidated in a field trial, when phage-impregnated feed was administered to ayu in a pond where the disease occurred naturally. The daily mortality of the fish decreased at a constant level (ca. 5% per day), and by the end of the 2-week study period, it was approximately one-third of the mortality rate in the phage-untreated group. As with the previous studies, no phage-resistant organisms and phage-neutralizing antibodies were detected (Park and Nakai, 2003).

13.4. PHAGE-ELICITED BACTERIAL LYSATES AS VACCINES

13.4.1. Using Phages to Produce Bacterial Vaccines

An intriguing approach to using phages in agribusiness (and potentially for human therapy) involves using them to prepare bacterial lysates that can be used as vaccines. The approach is as old as conventional phage therapy (i.e., the approach of using phages to lyse etiologic agents *in vivo*), and it was developed in d'Herelle's Pasteur Institute laboratory shortly after the 1917 publication of his milestone paper (d'Herelle, 1917) on the discovery of bacteriophages. Beginning in 1914, d'Herelle was working to prepare bacterial vaccines for the Allied armies fighting in World War I (Summers, 1999). At that time, bacterial vaccine therapy was a very important

research subject at the Pasteur Institute, and the Institute's director (Emile Roux, one of the developers of a very successful anti-diphtheria vaccine) strongly encouraged the Institute's staff to develop new vaccines and the methodologies needed for their preparation. As part of that effort, d'Herelle examined the ability of various *essences* to lyse pathogenic bacteria and to produce lysates effective as vaccines. Many exotic bacteriolytic substances were examined as the project progressed; e.g., during his work to develop a *Salmonella* vaccine, d'Herelle examined mustard, cinnamon, garlic, oregano, cloves, and thyme for their ability to lyse *Salmonella* (Summers, 1999). All of them lysed the bacterium, and d'Herelle (1916) reported that mustard-generated lysates protected mice against challenge with live *Salmonella* (interestingly, it was an extension of that study—trying to develop a vaccine against *Shigella*—that led to his co-discovery of bacteriophage in 1917; see Chapter 2). However, even though d'Herelle postulated that phages were immunostimulating agents (and actually referred to phage therapy as an "immunization;" see section 13.2.1), he never directly examined the efficacy of phage-elicited bacterial lysates as vaccines. The first investigator to study the possible active immunization property of phage-generated lysates was Tamezo Kabeshima, who was visiting d'Herelle's Pasteur Institute laboratory from Shiga's laboratory in Japan. Kabeshima (1919) used a phage-lysed *Shigella* preparation ("bactériolysat") to successfully immunize small laboratory animals in what was to become the first known use of phage lysates as protective vaccines. Several subsequent publications reported that preparations of phage-lysed bacteria were indeed very good immunizing agents whose protective effect was stronger than that of "regular" vaccines (e.g., vaccines prepared by heat- or chemical-inactivation of bacteria) (Arnold and Weiss, 1924; Larkum, 1929; Compton, 1928).

The underlying mechanism for the superior immunogenicity reported for phage-generated bacterial lysates is not clear, but it is possible that bacteriophage-mediated lysis is a more effective and gentler approach for exposing protective antigens of bacteria than are approaches used to prepare other "dead cell" vaccines. In this context, common methods used to inactivate bacterial pathogens for "dead cell" vaccines (e.g., heat-treatment, irradiation, and chemical treatment) may indeed deleteriously affect a vaccine's effectiveness by reducing the antigenicity of relevant immunological epitopes (Melamed et al., 1991; Holt et al., 1990; Lauvau et al., 2001). Interestingly, the idea that gentle lysis yields cell lysates possessing optimal immunogenicity was proposed by d'Herelle (1916). Using phage lysates as vaccines is likely to have continued unknowingly since the early experiments of Kabeshima, because most of the therapeutic phage preparations used as "direct antimicrobial agents" were contaminated with numerous bacterial antigens released from the lysed bacteria and, thus, in addition to their bacteriolytic effect, those phage preparations may also have inadvertently acted as vaccines.

13.4.2. Using Phage-Generated Bacterial Lysates to Prevent and Treat Animal Infections

Perhaps the best known phage-generated bacterial lysate currently available for sale in the United States is SPL, which is produced by Delmont Laboratories of Swarthmore, PA. SPL is prepared from broth cultures of at least two virulent strains of coagulase-positive *S. aureus* (types I and III) by lysing the bacteria with an excess of *S. aureus*-specific bacteriophages. The lysate contains cell wall and intracellular components released as a result of bacterial lysis, culture media ingredients, and viable bacteriophages. As described in Chapter 14 of this book, SPL was used to prevent or treat human infections in the United States during the period from the 1950s to the 1990s, but is currently solely used for veterinary applications. In one of the early publications describing the use of SPL, Esber et al. (1981) used a mouse model to evaluate the efficacy of SPL in preventing or treating S. *aureus* infections of animals. Treatment with SPL resulted in the survival of 80%–100% of the infected mice, compared to no survivors among the infected mice not treated with SPL. Survival was hypothesized to be due to enhancement of nonspecific immune resistance elicited by SPL-mediated activation of thymus-modulated lymphocytes and macrophages. In another study by Esber et al. (1985), SPL administration was noted to significantly increase the anti-staphylococcal IgG1, IgG2a, and IgG2b levels in the treated animals. Weekly injections of SPL have also been reported (Chambers and Severin, 1984) to be an effective treatment for chronic staphylococcal blepharitis in dogs; i.e., the preparation controlled the illness without any adverse side effects in the animals. Also, the simultaneous use of SPL and sodium oxacillin was reported (DeBoer et al., 1990) to be significantly ($P < 0.05$) more effective in treating idiopathic, recurrent, superficial pyoderma in dogs than was treatment with the antibiotic alone. However, not all reports have been similarly encouraging. For example, in at least one study (Giese et al., 1996), vaccination with SPL was not found to prevent the development of *S. aureus* blepharitis, phlyctenules, and catarrhal infiltrates in an experimentally infected rabbit model. Additional, rigorous studies are required to improve our understanding of what infections/clinical syndromes are most amenable to prophylaxis and treatment with SPL (and to prophylaxis and treatment with other phage-generated bacterial lysates) and the optimal dosing regimens, administration routes, and so forth.

13.4.3. Phages and "Bacterial Ghost" Vaccines

Using phage-encoded bacteriolytic enzymes, rather than viable bacteriophages, to prepare bacterial vaccines has recently been gaining increased attention, particularly in Western Europe (Szostak et al., 1990; Szostak et al., 1996; Szostak et al., 1997; Eko et al., 1994a; Mader et al., 1997; Panthel et al., 2003). Such preparations contain what are often called "bacterial ghosts," and they are obtained by using lysis gene *e* of bacteriophage φX174 to lyse various Gram-negative bacteria (noteworthy, bacteriophage φX174 has been injected into humans in the United States, during studies designed to determine the immune status of various immunocompetent and immunocompromised individuals, as discussed in Chapter 14). The genome of bacteriophage φX174 encodes a single lysis protein E—an outer membrane protein that contains 91 amino acid residues (see Chapter 7), which can form a 40–200 nm diameter pore in the bacterial cell wall, though which the bacterium's intracellular constituents are expelled (Schon et al., 1995; Witte et al., 1990; Witte et al., 1992). The general methodology for obtaining "ghost" preparations can be briefly outlined on the example of a recent study in which *H. pylori* pHPC38 "ghost" has been

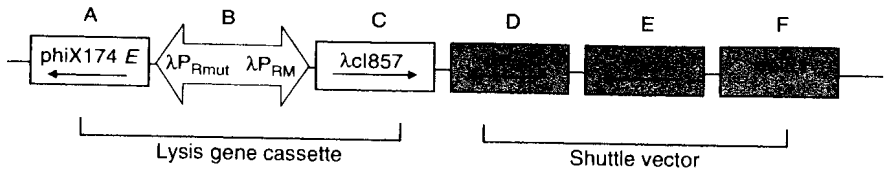

FIGURE 13.3 Schematic genetic map of *H. pylori* "ghost" pHPC38. (A) lysis gene *e* from bacteriophage φX174; (B) promoter region of λ phage; (C) ts-repressor; (D) chloramphenicol acetyltransferase gene; (E) replication in *H. pylori*; (F) replication in *E. coli* (adapted from Panthel et al., 2003).

prepared and examined for immunogenicity in mice by Panthel et al. (2003). The authors used the *E. coli-H. pylori* shuttle plasmid pHel2 to construct *H. pylori* lysis plasmid pHPC38, which they introduced into *H. pylori* strain P79 by conjugation or by natural transformation. The *H. pylori* transformants or transconjugants carrying it were selected on a solid culture medium containing chloramphenicol; the shuttle vector contains the chloramphenicol acetyltransferase gene, cat_{GC}, so including chloramphenicol in the medium selects bacterial colonies that contain the shuttle vector encoding the resistance-conferring cat_{GC} gene (Fig. 13.3). The ghost preparation was subsequently used to vaccinate BALB/c mice orally (using an initial dose followed by two booster doses at 7-day intervals); control mice received PBS. Three weeks after receiving the last dose of the *H. pylori* ghost preparation, the vaccinated and control mice were orally challenged with a high dose (10^9 CFU) of the wild-type *H. pylori* strain P79. The mice were sacrificed 4 weeks later, and gastric colonization with the challenge *H. pylori* strain was quantitated. Vaccination with the ghost preparation reduced the number of *H. pylori* in the gastric samples by ca. 1000-fold compared to the control group. The study did not determine the length of the protective effect; however, the data strongly suggested that a ghost vaccine may be an effective tool for preventing at least initial colonization of the gastric mucosa with *H. pylori*.

In theory, it should be possible to use a similar strategy to prepare bacterial ghosts of all Gram-negative bacteria, provided that the E lysis cassette can be introduced into the recipient by an appropriate vector, thus allowing tight repression and induction control of the *e* gene (Jalava et al., 2002). Indeed, ghosts of several Gram-negative bacteria have been prepared using E protein-mediated lysis, including *E. coli* (Blasi et al., 1985), *Salmonella* serotypes Typhimurium and Enteritidis (Szostak et al., 1996), *V. cholerae* (Eko et al., 1994a; Eko et al., 2003; Eko, et al., 1994b), and *Pasteurella multocida* and *P. haemolytica* (Marchart et al., 2003). Since the ghosts retain their outer membranes with their immunostimulatory lipopolysaccharide (LPS) endotoxin structure essentially intact (with the exception of the pores formed by the E protein), they should provide protection similar to that obtained using whole-cell, attenuated vaccines—but without the associated safety concerns (Jalava et al., 2002; Witte et al., 1990). One example of using ghost vaccine to prevent disease in agriculturally important animals is the recent work with the *Actinobacillus pleuropneumoniae* ghost vaccine and infection of pigs. *A. pleuropneumoniae* is a major respiratory pathogen responsible for severe morbidity and mortality in pigs; although conventional *A. pleuropneumoniae* vaccines are currently available, they decrease mortality but are ineffective in reducing morbidity. Hensel et al. (2000) used an aerosol infection-pig model to study the protective potential of an *A. pleuropneumoniae* (serotype 9, reference strain CVI 13261) ghost vaccine. Pigs were intramuscularly vaccinated with the ghost vaccine or with a formalin-killed *A. pleuropneumoniae* preparation. Two weeks later, the vaccinated pigs and nonvaccinated placebo controls were challenged with *A. pleuropneumoniae* (10^9 CFU) by aerosol inhalation. The outcome was evaluated by clinical, bacteriological, serological, and post-mortem examinations. The pigs in the control (untreated) group developed fever and pleuropneumonia after challenge with *A. pleuropneumoniae*; in contrast, the pigs in both treated groups were fully protected against clinical disease. Significantly, the ghost vaccine (but not the formalin-killed bacterial lysate) also prevented colonization of the respiratory tract with *A. pleuropneumoniae*. Treatment with the two vaccines also significantly increased the amounts of IgM, IgA, IgG (Fc'), and IgG (H+L) antibodies reactive with *A. pleuropneumoniae*. Interestingly, higher titers of IgG (Fc') and IgG(H+L) were observed in pigs treated with the formalin-killed vaccine than in pigs vaccinated with the ghost preparation; on the other hand, prevention of the carrier state in ghost vaccine-treated pigs coincided with a significant increase in serum IgA when compared to formalin-killed vaccine. A subsequent publication of a similar study (Huter et al., 2000) confirmed that immunization with a *A. pleuropneumoniae* ghost vaccine (but not with a formalin-inactivated preparation) prevented colonization of pig lungs with *A. pleuropneumoniae*.

Ghost vaccines have also been reported to prevent infections caused by *K. pneumoniae* (causes severe infections in various agriculturally important animals, including dairy cows, poultry, ostriches, etc.), *E. coli* 078:K80 (causes colibacillosis in poultry, and septicemia in calves, piglets and lambs), *P. multocida* (causes fowl cholera, and pneumonia in pigs and cattle), and other bacterial pathogens. Comprehensive reviews about bacterial ghost vaccines have been published (Szostak et al., 1990; Szostak et al., 1996; Szostak et al., 1997), including a recent review on bacterial ghosts as vaccine candidates for veterinary applications (Jalava et al., 2002). The significant amount of bacterial LPS endotoxin in bacterial ghost preparations/vaccines could potentially limit the use of this type of vaccine. However, several studies found that effective doses of the ghost vaccines did not elicit appreciable side effects in any of the animals examined. Moreover, the LPS component has been proposed to play a critical role in immune stimulation associated with ghost vaccines. For example, Szostak et al. (1996) reported a significant correlation between the endotoxic activity of bacterial ghost preparations, as determined by the *Limulus* amoebocyte lysate (LAL) assay, and their capacity to stimulate the release of PGE2 and TNFα in mouse macrophage cultures. In addition, bacterial ghosts prepared from *E. coli* O26:B6 and *Salmonella typhimurium* have been shown (Mader et al., 1997) to elicit dose-dependent antibody responses against bacterial cells and their corresponding LPS when administered intravenously to rabbits via a standard immunization protocol. No side effects were observed in any of the rabbits that received the ghost vaccines, in doses of <250 ng kg-1. Szostak et al. (1996) found that the

endotoxic activity of the bacterial preparations (analyzed by the LAL assay and the 2-keto-3-deoxyoctonate assay) correlated with the release of PGE2 and TNFα in mouse macrophage cultures and the endotoxic (i.e., fever) responses in rabbits—which suggests that the *in vitro* systems can be used to determine the potency of bacterial ghost vaccines (Mader et al., 1997).

At the present time, it is difficult to predict whether phage-generated bacterial lysates and/or ghost preparations will gain wide acceptance in veterinary medicine. Both approaches seem to be safe, effective, and relatively cost-efficient. However, phage lysates may have an advantage over ghost vaccines because they are simpler to prepare (e.g., they do not require the genetic engineering and optimal expression of *e* gene-containing constructs for bacterial lysis). The relative simplicity of preparing phage lysates compared to ghost preparations may be particularly important when complex vaccines need to be developed; e.g., vaccines against multiple serotypes of a given species when a vaccine based on one serotype does not provide adequate cross-protection across the species. The presence of viable phages may also serve as an additional efficacy-enhancing factor, increasing the effectiveness of a phage lysate via their antibacterial effect on the targeted bacterial pathogen.

13.4.5. Using Phage-Encoded Enzymes As Antibacterial Agents

In addition to the φX174-encoded E protein that has been used to prepare ghost vaccines, other phage-encoded enzymes have been used to lyse bacterial cells; e.g., the L protein of bacteriophage MS2 and the maturation protein A2 of phage Qβ (Bernhardt et al., 2001; Kastelein et al., 1982; Coleman et al., 1983), and the *B. amyloliquefaciens* phage endolysin. Also, some bacteriolysin-encoding genes and their applications have been patented; e.g., Auerbach and Rosenberg (1987) patented the use of cloned λ lysis genes as a method for bacterial cell disruption.

The applicability of the concept of using phage-encoded enzymes as antibacterial agents has been addressed in several recent studies. One example is the above-described use of the polysaccharide depolymerase encoded by ϕEa1 to attenuate the symptoms of fire blight in pear fruit inoculated with *E. amylovora* (Hartung et al., 1988). In another study, Loessner et al. (1998) cloned and sequenced the endolysin gene *plyTW* of *S. aureus* bacteriophage Twort (the gene encodes an ca. 53-kDa protein whose catalytic site is located in its amino-terminal domain). The cloned gene was over-expressed in *E. coli*, and the purified recombinant protein was shown to cleave staphylococcal peptidoglycan rapidly, thus suggesting that the endolysin may be an effective antibacterial agent against *S. aureus*. More recently, Gaeng et al., (2000) identified two endolysins (Ply118, an ca. 31-kDa L-alanoyl-D-glutamate peptidase, and Ply511, an ca. 37-kDa N-acetylmuramoyl-L-alanine amidase), encoded by the phage A118 (Loessner et al., 1995), which specifically hydrolyze the cross-linking peptide bridges in *Listeria* peptidoglycan, and they engineered a *Lactococcus lactis* strain that secreted both enzymes. This strain eliminated *L. monocytogenes* from dairy starter cultures used to produce cheese, which suggests that this approach may be of value in improving the safety of dairy products. More information about various lytic enzyme systems (including bacteriophage-encoded lytic enzymes) can be found in review articles by Dabora et al. (1990) and Young (1992), and in Chapters 7 and 12. Bacteria-encoded enzymes capable of lysing bacteria (e.g., lysostaphin and lysozyme) have been studied from the 1960s through the 1990s as potential therapeutic agents in numerous animal models and in at least one human patient (Schuhardt and Schindler, 1964; Stark et al., 1974; Gunn and Hengesh, 1969; Oldham and Daley, 1991; Nuzov, 1984; Nakazawa et al., 1966). However, the idea that phage-encoded enzymes could, on their own, be used for therapeutic purposes was not extensively discussed until relatively recently (Loeffler et al., 2001; Nelson et al., 2001; Schuch et al., 2002; Morita et al., 2001).

13.5. USING BACTERIOPHAGES IN THE FOOD PRODUCTION CHAIN

13.5.1. General Considerations

Foodborne illnesses of microbial origin are serious food safety problems worldwide. The Center for Disease Control and Prevention (CDC) estimates that about 76 million cases of foodborne diseases (of which ca. 5000 are fatal) occur each year in the United States alone—and bacteria account for about 72% of all deaths associated with foodborne transmission (21% are due to parasitic infections and 7% are caused by viruses) (Mead et al., 1999). Among cases of foodborne illness, the leading causes of death are *Listeria*, *Toxoplasma*, and *Salmonella*, which together are responsible for more than 75% of foodborne deaths caused by known pathogens. In addition, Shiga toxin-producing *E. coli* (including strains of the 0157:H7 serovar) have recently emerged as a major food safety problem (in particular in ground beef) and they have caused several major outbreaks of disease with many fatalities. Also, several *Brucella*, *Yersinia*, *Shigella*, and other bacterial species are significant causes of foodborne disease, with *Campylobacter* species causing the largest number of cases in the United States (*C. jejuni* causes approximately 2.4 million illnesses/year in the United States) (Mead et al., 1999).

The epidemiology of foodborne diseases due to bacterial pathogens varies among the pathogens, as do the routes by which various bacteria contaminate food products. Some bacteria (e.g., *L. monocytogenes*) are environmental pathogens that usually contaminate foods in food processing/packaging plants. Other bacteria (e.g., *Salmonella* and *Campylobacter*) are part of the normal intestinal flora of many animals, and they contaminate foods during the slaughter or carcass processing cycle. Using bacteriophages to reduce contamination of foods with various bacterial pathogens will require an in-depth understanding of the epidemiology of the pathogen against which the phage preparation is to be used and the identification of critical intervention points in the processing cycle where phage application will be most beneficial (Stone, 2002). In this context, three possible areas of application for phage technology may be loosely identified: (i) Phages may be used to reduce intestinal colonization of live, agriculturally important animals that normally carry bacteria which present a foodborne disease risk; (ii) Phages may be applied directly onto raw foods, or onto environmental surfaces in raw food processing facilities, to reduce the levels of foodborne pathogens in raw foods; and (iii) Phages may be applied directly onto ready-to-eat foods (REF), or onto environmental surfaces in processing facilities for

REF, to reduce the levels of pathogenic bacteria in REF. Additional applications and combinational approaches (e.g., when phages are used in live animals and also during the processing) also can be utilized. At the present time, no such applications are utilized in industrial settings. However, as described below, the results of some published studies examining their efficacy suggest that the approaches have merit.

13.5.2. Using Phages to Reduce Contamination of Livestock with Foodborne Pathogens

Many bacterial pathogens capable of causing foodborne illness in humans are part of the normal flora of the gastrointestinal tract (GIT) of agriculturally important domesticated animals (cows, sheep, poultry, etc.). During the animals' slaughter and processing, there are multiple opportunities for the bacteria to contaminate the raw carcass, which increases the risk of the bacteria being subsequently ingested by humans and eliciting disease. Thus, approaches that eliminate or reduce the levels of foodborne pathogenic bacteria in foods have a potential to significantly improve the safety of food products. The use of bacteriophages may provide one such option.

As noted earlier in this chapter, several investigators have shown that oral administration of phages reduces the amount of targeted bacteria in, and reduces fecal shedding of the bacteria from, the intestines of the treated animals (Smith and Huggins, 1983; Smith et al., 1987a; Berchieri et al., 1991). The studies were not designed with food safety in mind—rather, they were conducted to determine whether oral administration of phages has a prophylactic effect on bacterial diseases in various animal species. Nevertheless, they provide indirect supporting evidence that oral administration of phages may indeed be effective in the context of food safety. The idea that phage-mediated reduction of intestinal colonization with pathogenic bacteria or of shedding of pathogenic bacteria may improve food safety has been advanced relatively recently (Brabban et al., 2003; Raya et al., 2003; Kudva et al., 1999). The preliminary data generated during these recent studies support the early observations already referenced and suggest that the approach has merit. For example, a single oral dose of a highly concentrated (4×10^{11} PFU) of an *E. coli* O157:H7-specific phage preparation recently was reported (Raya et al., 2003) to elicit, by two days post-administration, a marked reduction in intestinal levels of *E. coli* O157:H7 in sheep experimentally colonized with the bacteria (10^{10} CFU) three days prior to phage treatment. The authors proposed that "these results suggest that the protective effect of bacteriophages against *E coli* O157:H7 may contribute significantly to reducing the incidence of human infection if used in a preventive manner" (Raya et al., 2003). Although their initial findings were encouraging, the authors acknowledged the need to perform additional studies addressing several critically important issues not examined during their initial studies.

One such issue is the practical applicability of using phages to eliminate or significantly reduce the levels of foodborne pathogens in the GIT of agriculturally important animals. The composition of an animal's normal intestinal flora is very complex, and eliminating intestinal colonization by various bacteria (particularly if the bacteria are part of the GIT's normal flora) has proven to be very difficult. Thus, it is doubtful that phages can completely eradicate bacteria that commonly reside in the GIT of domesticated livestock and possess the ability to be foodborne pathogens of humans. Also, the value of reducing the levels of pathogenic foodborne bacteria in the GIT of domesticated livestock in terms of both environmental contamination and the impact on improving the safety of animal-based foods has yet to be determined. Furthermore, although phage resistance did not seem to be a major problem during short-term phage therapy studies in animals and fish (Smith and Huggins, 1983; Bull et al., 2002; Berchieri et al., 1991; Park and Nakai, 2003), long-term direct and continued exposure of phages and their targeted bacteria in the GIT may provide fertile grounds for selecting phage-resistant mutants (see Chapter 14). Thus, there may be advantages to using phages at an "epidemiological endpoint;" i.e., when cycling of the pathogen and phage in the environment and/or mammalian host does not occur or is minimized. An intriguing approach based on that idea was developed by Taylor et al. (1958), who injected phages into the interior of fertile eggs prior to incubation. Their hypothesis was that the phages would lyse the bacteria in the eggs, which in turn would improve the chick' hatch-rate—and some evidence of that effect was presented by the authors. For example, the hatch-rate of eggs experimentally infected with *Salmonella* Chittagong was 47% vs. 70% in phage-untreated and phage-injected eggs, respectively. The difference in rates was similar for other *Salmonella* serotypes (e.g., the hatch rate with eggs infected with *Salmonella* Pullorum was 44%, compared to 77% with eggs infected with the same bacterium and treated with a specific phage preparation). The authors did not discuss the potential food safety benefits of their observations, and the effect of treating the infected eggs with phage was solely evaluated by comparing the hatch rates; no rigorous microbiological examination of the eggs and hatched chicks was performed. However, it is tempting to speculate that, if the bacteriophages were effective in reducing the levels of pathogenic salmonellae present in the eggs, the approach may be of value in preventing human diseases caused by egg-borne bacterial pathogens. A benefit of the approach is that, since the phages injected into eggs will primarily be exposed only to bacteria in the eggs, a broad selective pressure against susceptibility to bacteriophages is unlikely to develop. Other possible food production uses for bacteriophages which are not likely to result in a selective pressure stimulating the emergence of phage-resistant bacteria, are discussed below.

13.5.3. Using Phages to Reduce Contamination of Raw Foods with Foodborne Pathogens

Another way that therapeutic phage may be used to improve food safety is to apply them directly onto raw food products or onto environmental surfaces in raw food processing facilities, in order to reduce the levels of foodborne pathogens in raw foods. As noted above, one of the most critical elements in that type of approach is to determine the optimal point(s) of intervention, or the time/step in the food processing cycle where exposure to phages is most beneficial and also minimizes the selective pressure for phage-resistance. A possible approach, suggested by the above-described studies of Taylor et al. (1958), would be to inject, rinse, or spray fertilized eggs with bacteriophage solutions before transferring them into incubators. That approach may be suitable for several egg-borne bacterial pathogens;

e.g., *Salmonella* and *C. jejuni*. Those bacteria often are present on the surface of fertilized eggs; because the temperature and humidity in incubators promote bacterial multiplication, the bacteria may increase in numbers during incubation, and chicks may become infected as they peck out of the eggs. Therefore, spraying phages onto the surface of eggs before transferring them into incubators may provide a gentle means of minimizing *Salmonella* contamination of eggs (and, perhaps, for increasing hatch-rates) which may subsequently lead to reduced levels/incidence of *Salmonella* in hatched chicks and to a reduction in *Salmonella* contamination of poultry products.

Alternatively, phages could be sprayed onto the chicken carcasses after post-chill processing (e.g., after the chlorine wash in chiller tanks in poultry processing plants in the United States, or after processing through air chillers in Europe). At that point, contamination with the usual foodborne bacterial pathogens should be minimal as a result of current *Salmonella* and *Campylobacter* reduction practices employed by all major poultry producers, and applying phages at that point in the processing cycle may provide a final means of product cleanup. In that context, Atterbury et al. (2003) recently reported that anti-*Campylobacter* bacteriophages are common commensals of retail poultry in the United Kingdom, and could survive commercial poultry processing procedures—which suggests that their application in real-life industrial settings may be technically feasible. Another important advantage of applying phages at this stage is that, since phages will not be carried to loci where they can readily be exposed to *Salmonella* or *Campylobacter* for a long period of time (e.g., to chicken houses), the risk of these bacteria developing resistance against the phages will be greatly reduced. Laboratory confirmation that applying phage onto chicken skin may be of value in reducing bacterial contamination has recently been published (Goode et al., 2003). The authors demonstrated that applying *C. jejuni*-specific phages onto chicken skin experimentally contaminated with the bacteria elicited a 10- to 100-fold reduction in the number of contaminating bacteria. For example, applying ca. 10^6 PFU of *C. jejuni* typing phage NCTC 12673/cm^2 of chicken skin reduced the levels of the test, contaminating *C. jejuni* strain on chicken skin by 95% ($t = 2.5$; $P = 0.04$). For *Salmonella* (which survived on untreated chicken skin better than did *C. jejuni*), the application of phages resulted in a significant ($P < 0.01$), ca. 99% reduction on phage-treated samples compared to the untreated controls. When the level of initial *Salmonella* contamination was low, phage treatment resulted in the samples being free of any recoverable *Salmonella* Enteritidis test strain organisms.

The results of these studies suggest that the approach of spraying specific phage preparations onto poultry carcasses after post-chill processing is efficacious in reducing carcass contamination with foodborne pathogens. The practical applicability of the approach may be complicated by narrow host range of phages—which may be a particular problem with *Salmonella*, a highly heterogeneous species containing more than 2400 serotypes (Popoff et al., 2000). However, it should be possible to target strains or serotypes known to be responsible for the majority of human illnesses, or which have increased virulence, such as *S.* Typhimurium definitive phage type 104 (DT104), which have rapidly emerged as major foodborne pathogens worldwide (Poppe et al., 1998). Assuming some flexibility from the regulatory agencies, it should also be possible to customize the phage preparations to provide coverage against additional *Salmonella* serotypes, or specific "problem" strains or serotypes in a flock or commercial production facility.

13.5.4. Using Phages to Reduce Contamination of Ready-to-Eat Foods with Foodborne Pathogens

An extension of the above-described approach is to apply specific phages onto various REF, in order to eliminate, or reduce the amount of, specific bacterial pathogens on those foods. Contamination of REF with foodborne pathogens is a potentially much more serious problem than is contamination of raw foods that are usually cooked before consumption, which can dramatically reduce the levels of pathogens in them. In contrast, and as the name implies, REF are often consumed without any additional processing; therefore, if they are contaminated with pathogens, the risk of human disease is high.

The value of using bacteriophages to eliminate or reduce the number of foodborne pathogens on various REF was first examined with fresh-cut fruits and vegetables by Leverentz et al. (2001). The authors examined the ability of phages to reduce experimental *Salmonella* contamination of fresh-cut melons and apples stored at various temperatures likely to be encountered in real-life settings. Directly applying the phage preparation (25 μl of 2×10^8 PFU/ml per fruit slice, applied by pipette) to the experimentally contaminated fruit reduced *Salmonella* populations by ca. 3.5 logs on honeydew melon slices stored at 5°C and 10°C, and by ca. 2.5 logs on slices stored at 20°C (Fig. 13.4), which was better than that achieved using commonly used chemical sanitizers. However, the phage preparation was less effective on fresh-cut Red Delicious apples than on the honeydew melon slices. Significantly, the titer

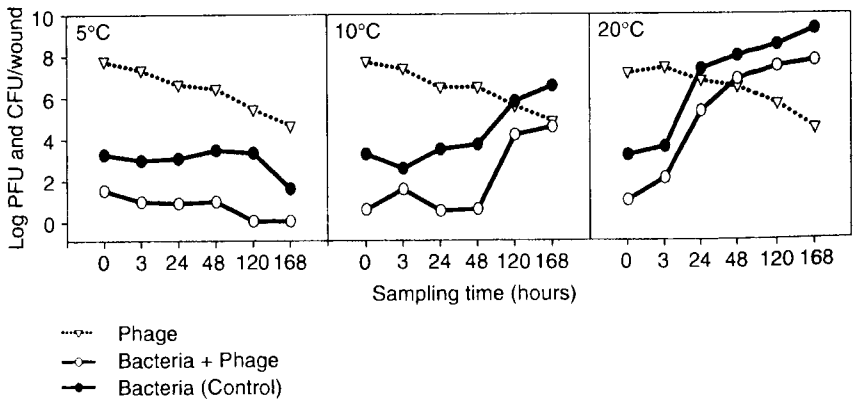

FIGURE 13.4 Populations of *Salmonella* Enteritidis on honeydew melon slices treated with a mixture of *Salmonella*-specific bacteriophages, and stored at 5°C, 10°C, and 20°C for 168 hours. Dotted line shows titer of the phage mixture re-isolated from slice of fruit stored at various temperatures (from Leverentz et al., 2001, reprinted with permission from *Journal of Food Protection*. Copyright held by the International Association for Food Protection, Des Moines, Iowa, U.S.A.).

of the phage preparation remained relatively stable on the melon slices, while on apple slices, it decreased to nondetectable levels in 48 h at all temperatures tested. The authors hypothesized that inactivation of the phages, possibly by the apple slices' more acidic pH (pH 4.2 versus pH 5.8 for melon slices), contributed to the inability of the phage preparation to reduce *Salmonella* contamination significantly in the apple slices. They also suggested that using higher phage concentrations and/or low-pH-tolerant phage mutants might be effective ways to increase the efficacy of phage treatment of fresh-cut produce with a low pH. The value of those suggested approaches has not yet been evaluated.

In another *Salmonella*-related study (Whichard et al., 2003), Felix O1 bacteriophage and its mutant possessing increased *in vitro* lytic activity against *Salmonella* Typhimurium DT104 have been used to treat chicken frankfurters experimentally contaminated with *Salmonella* Typhimurium DT104. The wild-type phage and the mutant phage elicited a 1.8 log and 2.1 log reduction, respectively, in *Salmonella* levels, compared to the levels in the untreated frankfurters ($P = 0.0001$). Although the mutant phage appeared to be more effective than the wild-type phage in lysing *Salmonella* lawns growing on a bacteriologic culture medium, the difference in the efficacies of the two phages in reducing the *Salmonella* content of the contaminated frankfurters was not statistically significant ($P = 0.5$). A similar approach—i.e., using specific phages to eliminate or significantly reduce the levels of contaminated bacteria on fresh-cut fruits and vegetables—also has been noted to be under investigation for *E. coli* 0157:H7 (Kudva et al., 1999).

Leverentz et al. (2003) recently reported the results of their second study examining the efficacy of phages, alone and in combination with the bacteriocin nisin, in reducing the levels of *L. monocytogenes* on experimentally contaminated melons and apples. The phage mixture, applied by pipeting or spraying, reduced *L. monocytogenes* populations by 2.0 to 4.6 log units on honeydew melons compared to controls treated with phage-free buffer. As previously observed with *Salmonella* (Leverentz et al., 2001), the reduction was less profound on apples (an ca. 0.4 log decrease compared to controls) than it was on melons. Using the phage preparation in combination with nisin decreased *L. monocytogenes* populations by up to 5.7 log units on honeydew melon slices and by up to 2.3 logs on apple slices, compared to the phage-untreated controls. In another parallel to their earlier *Salmonella* study, *L. monocytogenes*-specific phage titers were stable on the melon slices, but they declined rapidly on the apple slices. The effectiveness of the phage treatment depended on the initial concentration of *L. monocytogenes*; i.e., the greatest reductions were achieved with the highest phage/*L. monocytogenes* ratios. Since real-life contamination of fresh-cut produce with *L. monocytogenes* is not likely to occur at the artificially high bacterial levels used during the experiments (e.g., 2.5×10^5 and 2.5×10^6 CFU of *L. monocytogenes* per 0.785 cm^3 of fruit), the authors suggested that the phage preparation may be even more effective in real-life settings than during their experiments. Spray application of phages reduced the bacterial numbers at least as much as the earlier pipette application, which is of importance for commercial applications. Phage spray treatment has also been shown (S.L. Burnett, personal communication) to reduce the levels of *L. monocytogenes* on sliced cooked whole muscle cuts (red meat and poultry), sliced cooked cured whole muscle cuts (red meat and poultry), and whole muscle cuts (red meat and poultry) by at least 10-fold (and, on several foods, by more than 100-fold) when the products were stored refrigerated. In view of the recent USDA and FDA estimate (Anonymous, 2003) that a tenfold pre-retail reduction in contamination with *L. monocytogenes* would reduce the annual number of *L. monocytogenes*-caused deaths in the elderly population in the United States by nearly 50%, the aforementioned ability of specific phages to reduce significantly these levels on various REF foods may have very practical significance.

These studies suggest that using phages to reduce contamination of foods with various pathogenic bacteria is effective for at least some types of foods, and that the successful use of phages will be a matter of using the right phage in the right place and in the right concentration. However, being effective is only the starting point in the process of making this approach viable, and many additional important issues will have to be addressed before phages can be used to improve food safety in real-life settings. For example, phage preparations added to foods must meet stringent requirements for purity, which will necessitate the development of commercially viable protocols for the large-scale production of purified phage. Also, at the present time, it is not clear what the regulatory strategy will be for such phage-based products; e.g., it is unclear whether they will be treated as "direct food additives" (i.e., ingredients that have a long-lasting effect on the product; e.g., lactoferrin) or as "secondary direct food additives" (i.e., ingredients that do not have a long-lasting effect on the foods; e.g., ozone). Finally, consumers' acceptance of the idea that phages (i.e., kinds of viruses) may be added to their food is another unknown. Since the great majority of people are not aware of the naturally high prevalence of phages in the foods they consume daily (see section 13.5.5 below), they may be concerned by the idea of adding phages to some of their foods, even if its purpose is to eliminate harmful bacteria and make the foods safer for human consumption.

13.5.5. Prevalence of Bacteriophages in Foods and Other Parts of the Environment

As described in detail in Chapter 6, bacteriophages are the most ubiquitous and most diverse living entitites on Earth. The total number of phages on Earth is estimated to be 10^{30} to 10^{32}, and they are abundant in saltwater and freshwater, soil, plants, and animals; in humans, phages can be found on skin, in the GI tract, and in the mouth (Rohwer, 2003; Brüssow and Hendrix, 2002; Bergh et al., 1989; Gill et al., 2003; Bachrach et al., 2003; Yeung and Kozelsky, 1997). Bacteriophages also have been isolated from drinking water (Lucena et al., 1995; Armon et al., 1997; Armon and Kott, 1993; Grabow and Coubrough, 1986) and a wide range of food products, including ground beef, pork sausage, chicken, farmed freshwater fish, common carp and marine fish, oil, sardines, raw skim milk, and cheese (Hsu et al., 2002; Whitman and Marshall, 1969; 1971; Kennedy et al., 1986; Kennedy et al., 1984; Gautier et al., 1995). To give just a few examples, bacteriophages were recovered from 100% of examined fresh chicken and pork sausage samples and from 33% of delicatessen meat samples analyzed by Kennedy et al. (1984). The levels ranged from 3.3 to 4.4×10^{10} PFU/100 g of fresh chicken, up to 3.5×10^{10} PFU/100 g of fresh pork, and up to 2.7×10^{10} PFU/100 g of roast turkey breast samples. In another study

(Kennedy et al., 1986), samples of fresh chicken breasts, fresh ground beef, fresh pork sausage, canned corned beef, and frozen mixed vegetables were examined for the presence of coliphages. Although only three ATCC strains of *E. coli* were used as indicator host strains, coliphages were found in 48% to 100% of the various food samples examined. Several other studies have suggested that 100% of the ground beef and chicken meat sold at retail contain various levels of various bacteriophages (Hsu et al., 2002; Kennedy et al., 1986; Tierney et al., 1973).

Meats and vegetables are not the only foods in which the presence of phages has been well documented. For example, Gautier et al. (1995) recently reported that 50% of the Swiss cheese samples they analyzed contained phages lytic for *Propionibacterium freudenreichii* (dairy propionbacteria are used in the production of Swiss cheese because of their ability to produce the characteristic, desired flavor). The number of bacteriophages varied from 10 to 10^6 PFU/g of cheese, and the authors thought it was likely that their data was an underestimate, since only a few indicator strains were used during the screening. Bacteriophages have also often been found in various seafoods. For example, during studies examining the value of bacteriophages as surrogate markers for detecting pollution in shellfish, *B. fragilis* bacteriophages often were isolated from black mussels (Lucena et al., 1994). Bacteriophages also are often found in animal feed. In a recent study from Texas A&M University (Maciorowski et al., 2001), male-specific and somatic coliphages were detected in all animal feeds, feed ingredients, and poultry diets examined, even after the samples were stored at –20°C for 14 months.

The above data indicate that naturally occurring bacteriophages are commonly consumed by humans and animals; the daily ingestion of phages may be an important natural strategy for replenishing the phage population in the GIT and for regulating the colon's microbial balance. However, no accurate estimate of the amount of phage ingested daily by an average person is available at the present time. It is likely that only a small portion of the viable phage in foods—including phages intentionally added to foods, if any, to reduce the levels of foodborne pathogens—actually are or will be consumed by humans, because many commonly used food processing practices are detrimental for phage viability; e.g., microwaving for ≥1 min and/or boiling for ≥2 min reduces the levels of viable phage in various foods by >99.9% (A. Sulakvelidze, unpublished data). Thus, it is possible that most of the food-delivered phage found in the human GIT come from eating unprocessed foods. Furthermore, at least some of the ingested viable phage are likely to be killed by the acidic conditions in the stomach; probably only a small percent actually are capable of "colonizing" the intestines. The length of time that particular phage persists in the intestines also is unknown at the present time. However, data concerning the prevalence of phages in human feces and wastewater samples may provide some insight into their prevalence in the human GIT and in wastewater and sewage.

Phages capable of lysing *E. coli, B. fragilis*, and various *Salmonella* serotypes have been isolated from human fecal specimens in concentrations as high as 10^5 PFU/100 g of feces (Calci et al., 1998; Cornax et al., 1994; Furuse et al., 1983a; Kai et al., 1985). Importantly, because of the specificity of phages, employing a specific bacterial host for phage enumeration will only enable enumeration of the phages that can infect that particular host strain; thus, the value of such an approach for enumerating the entire phage population is limited. An interesting approach to counteract this limitation has recently been explored by Breitbart et al. (2003). The authors used partial shotgun sequencing to perform metagenomic analyses of an uncultured viral community in human feces. Bacteriophages were found to be the second most abundant category (after bacteria) in the uncultured fecal library, containing an estimated 1200 diverse genotypes, 80% of them siphoviridae. It is likely that further optimization of this approach, and further development of microarray-based technologies, will provide much information about the diversity of phage populations, and the peculiarities of phage-bacterial interactions, in the GIT.

A yet-unidentified proportion of the phages "colonizing" the human GIT is continuously being released into the environment via wastewater. Many studies have been performed to determine the amounts of various bacteriophages in wastewater samples (Lasobras et al., 1999; Hantula et al., 1991; Puig et al., 1999; Ketranakul and Ohgaki, 1989; Cornax et al., 1990; Havelaar and Hogeboom, 1984; Moce-Llivina et al., 2003; Leclerc et al., 2000; Duran et al., 2002; Furuse et al., 1981; 1979; Osawa et al., 1981b; Tartera and Jofre, 1987; Tartera et al., 1989), and some of them have been recently reviewed (Leclerc et al., 2000). To give just a few examples, one study (Lasobras et al., 1999) determined the levels of somatic coliphage, F-specific RNA phage, and *B. fragilis* phage in two water treatment plants in Spain. The protocols employed for phage isolation specifically focused on enumerating phage in sludge solids rather than on determining the total amounts of phage in wastewater samples. Also, it is likely that a proportion of the phage in the samples were not detected because of the methodological approaches employed; for example, sludge samples were centrifuged for a brief period of time before initiating phage isolation, supernatant fluids (possibly containing large amounts of phage) were discarded, and only the harvested sediments were processed for phage enumeration. The concentration of phage in the samples analyzed was still found to be fairly high (e.g., ca. 10^5 PFU of somatic coliphage/g-1 were recovered from primary sludge samples obtained from a biological treatment plant). Another study from Spain, by Puig et al. (1999), determined the prevalence of *B. fragilis* phage and *Salmonella* serotype Typhimurium phage in urban sewage samples and in wastewater samples from animals. Bacteriophage were isolated from all of the examined urban sewage samples and from 39%–100% of slaughterhouse wastewater samples (the percentage of positive samples varied depending on the host strain used for phage isolation). Bacteriophage concentrations varied depending on the samples, and they generally were higher in urban water samples than in samples from slaughterhouses. Similar observations were reported in a recent paper by Leclerc et al. (2000), in which the authors estimated daily per capita loadings of male-specific bacteriophage for various animal species. The authors also estimated the amounts of these phage released into the environment (PFU d-1) per animal host. The numbers ranged from a low of 9×10^2 PFU/day (canine) to a high of 2×10^7 PFU/day (horse). The sewage effluent loadings ranged from 1.1×10^{12} PFU/day to 2.0×10^{13} PFU/day for the same male-specific coliphage. Phages have also been commonly isolated from wastewater in Japan, Korea, and other countries (Furuse et al., 1979, 1981; Furuse et al., 1983a; Furuse et al., 1978; Furuse et al., 1983b; Havelaar et al., 1986; Havelaar et al., 1990; Kai et al., 1985; Osawa et al., 1981a; Osawa et al., 1981b).

As noted above, the amounts of phages released from human GIT into the environment have not been rigorously determined, but some estimates can be made. For example, Puig et al. (1999) reported that the average concentration of F-specific phages for *Salmonella* serotype Typhimurium WG49 in sewage from 13 locations in 7 countries in Europe was ca. 7.7×10^3 PFU/ml (Table 13.3). The average person in the United States generates approximately 135 gallons, or 511 liters, of sewage per day (Anonymous, 1972). Thus, assuming that the average number of F-specific phages in sewage in the United States is similar to that in Europe, the number of F-specific phages in raw sewage attributable to a single individual in the United States may be calculated as follows: 7.7×10^3 PFU/ml $\times$ 511 L. of sewage/person/day = 3.9×10^9 of phages specific for *Salmonella* serotype Typhimurium strain WG49 shed per person, per day. According to the U.S. Bureau of the Census, the resident population of the United States is approximately 292 million (as of October, 2003); thus, the amount of F-specific phages in U.S. sewage would be estimated at a mind-boggling 1.1×10^{18} PFU per day. Of course, this is a very rough estimate, based on many possibly incorrect assumptions. For example, it assumes that the F-specific phages do not multiply in sewage, which may be an appropriate assumption (based on previously published literature (Novotny and Lavin, 1971; Havelaar et al., 1990)) and that the phages' life-cycle in sewage is 24 h or less (which is not the case for the F-specific phages, shown (Lasobras et al., 1999) to remain viable in sewage samples for at least several days). Significantly, the above estimate is based on the number of only F-specific phage in sewage, and it does not account for any other phages that are also likely to be present there. Similar estimates based on phage prevalence data obtained during studies of two water treatment plants in Spain (Lasobras et al., 1999) suggest that the amount of somatic coliphages shed per person per day may be even higher than that of the F-specific phages. On the other hand, the number of *B. fragilis* bacteriophages shed per person per day is much lower than the amount of the F-specific phages and coliphages (Lasobras et al., 1999). The observed difference in prevalence between the *B. fragilis* phages and the F-specific phages and somatic coliphages may be due to a lower prevalence of *B. fragilis* phages in the human GIT. Another possible explanation for the observed difference might be that the methodological approach employed to enumerate the phages in sewage samples may have enabled better enumeration of the coliphages and F-specific phages than the phages specific for the more fastidious, anaerobic *B. fragilis*. However, more rapid inactivation of *B. fragilis* bacteriophages in sewage (compared to coliphages and F-specific phages) probably is not a contributing factor, since *B. fragilis* phages and somatic coliphages have been reported (Lucena et al., 1994) to have relatively low decay rates in sewage, the lowest among all phages examined.

13.5.6. Food Spoilage and Bacteriophages

Perhaps one of the least explored applications of bacteriophages in agribusiness is their possible efficacy in reducing food spoilage caused by various bacteria. Food spoilage involves a complex sequence of events involving the interaction of a combination of microbial and biochemical activities (reviewed in Borch et al., 1996; Huis in 't Veld, 1996). Microorganisms are the major cause of spoilage of most food

TABLE 13.3
Levels of *B. fragilis*- and F-specific Phages in Urban Sewage and Animal Wastewater Samples from Various Countries (adapted from (Puig et al., 1999))

Country	Bacteriophage Level (PFU/ml)					
	B. fragilis HSP40[a]		*B. fragilis* RY2056[a]		*S.* Typhimurium WG49[a]	
	Urban Sewage	Animal Wastewater	Urban Sewage	Animal Wastewater	Urban Sewage	Animal Wastewater
The Netherlands	1	0	4.6×10^2	3.4×10^3	5.1×10^3	3.0×10^4
The Netherlands	2.6	0	7.7×10^2	8.6×10^4	7.6×10^3	3.8×10^3
Ireland	1.4	0	3.0×10^2	0	4.6×10^3	4.8×10^2
Ireland[b]	1.6	0	4.4×10^2	0	6.9×10^3	1.0×10^1
Austria	8.5	ND	8.1×10^2	ND	1.6×10^3	ND
Austria	0.5	ND	6.1×10^2	ND	2.2×10^3	ND
Portugal	0.4	0	1.8×10^2	0.8	1.8×10^4	2.2×10^2
Portugal	0.1	ND	1.0×10^2	ND	3.8×10^4	ND
Portugal	0	0	1.0×10^2	ND	5.5×10^3	ND
Germany	2.2	0	7.8×10^2	1.3	4.8×10^3	5.7×10^3
Germany	1.3	ND	6.0×10^2	ND	2.2×10^3	ND
Sweden	0.9	ND	2.2×10^1	ND	1.9×10^3	ND
France	3.1×10^1	ND	2.3×10^2	ND	1.2×10^3	ND
South Africa	1.1×10^2	0	1.8×10^2	0	1.2×10^4	2.0×10^2
South Africa	4.5×10^2	0	ND	0	5.9×10^4	5.0×10^3
South Africa	1.2×10^2	ND	5.4×10^2	ND	1.7×10^4	ND
South Africa	2.3×10^2	ND	5.0×10^2	ND	2.4×10^4	ND

[a]Host strains used for phage enumeration
[b]The number of phages indicated for animal wastewater samples are for Denmark
ND—Not determined

products, and 25% of all post-harvest foods have been estimated to be lost due to microbial-elicited food spoilage (Gram and Dalgaard, 2002). However, only a few members of the microbial community, so called *specific spoilage organisms* (SSO), give rise to the offensive aromas and flavors associated with food spoilage. SSO are found in various types of foods, and various bacterial species are responsible for the different stages of food deterioration (Gram and Dalgaard, 2002; Samelis et al., 2000; Gamage et al., 1997; Jay et al., 2003).

Numerous approaches have been examined and used/are being used to reduce the number of SSO in foods, including hydrodynamic pressure processing, gamma irradiation, and pasteurization (Williams-Campbell and Solomon, 2002; Gamage et al., 1997; Roberts and Weese, 1998; Gould, 2000). Early attempts to use antibiotics to extend the shelf life of various foods were successful (Bernarde and Littleford, 1958); however, the use of antibiotics in agribusiness is not an optimal approach because it may promote the emergence and spread of antibiotic-resistance among various bacterial species (see section 13.2.4). An alternative to antibiotics may be disinfecting solutions and bacteriocins; e.g., nisin and lysozyme have been used, with some success, to control meat spoilage (Nattress et al., 2001; Thomas et al., 2002). Also, lactic acid bacteria have been used to inhibit the growth of pathogenic bacteria and SSO in various foods (Hernandez et al., 1993). However, lactic acid bacteria do not eliminate SSO, but, rather, inhibit their growth by competing for nutrients in the meat being treated. Moreover, lactic acid bacteria may contribute to the middle and late stages of food spoilage (Leroi et al., 1998). Therefore, biocontrol agents that do not contribute to food spoilage and can specifically target the SSO—without disturbing the normal, beneficial microflora of foods—may be an attractive modality for improving the shelf life of various food products. Bacteriophages seem to fit these requirements; however, the idea of using phages to reduce the levels of SSO in various foods has not been rigorously pursued and only a few publications are available on the subject.

One of the first publications examining the ability of phages to extend the shelf life of foods is the study by Ellis et al. (1973). The authors demonstrated that *P. fragi*-specific bacteriophages (originally isolated from ground beef (Whitman and Marshall, 1971) reduced the number of *P. fragi* in refrigerated raw milk and increased the milk's shelf life. More recently, the ability of phages to reduce beef spoilage was evaluated by Greer et al. (1990). Although the authors did not observe an appreciable extension of the shelf life of the examined beef products, they only targeted one bacterial species, which probably was not the major SSO responsible for beef spoilage. Indeed, when the same group of authors specifically targeted *B. thermosphacta* (the species known to be responsible for the development of unpleasant odors in spoiled pork tissues), the storage life of the meat increased from 4 days in the controls to 8 days in the phage-treated samples (Greer and Dilts, 2002). Similar results were reported for beefsteaks; i.e., the application of *Pseudomonas*-specific phages almost doubled the steaks' shelf-life (Greer, 1986). Thus far, the ability of bacteriophages to increase the shelf life of seafood products has not been rigorously addressed (Delisle and Levin, 1969).

The above-cited publications suggest that bacteriophages specifically targeting SSO can effectively reduce the number of SSO on various foods and extend their shelf life. However, the practical applicability of that approach is not clear. For example, given the narrow specificity of phages and the complexity of the bacterial flora involved in food spoilage, it may be challenging to develop effective anti-SSO phage preparations for industrial use. The problem may be further compounded by the fact that the precise identity of SSOs still has not been clearly determined for many foodstuffs. These, however, are not insurmountable tasks, and should become increasingly realistic as our understanding of the microbiology of food spoilage of various foodstuffs improves as the result of the ongoing scientific research in the field.

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