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# Bacteriophage P100 for control of *Listeria monocytogenes* in foods: Genome sequence, bioinformatic analyses, oral toxicity study, and application

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**Abstract**

*Listeria monocytogenes* is an opportunistic foodborne pathogen responsible for Listeriosis, a frequently fatal infection. This investigation represents a comprehensive approach to characterize and evaluate the broad host range, strictly virulent phage P100, which can infect and kill a majority of *Listeria monocytogenes* strains. First, the complete nucleotide sequence (131,384 basepairs) of the genome of P100 was determined, predicted to encode 174 gene products and 18 tRNAs. Bioinformatic analyses revealed that none of the putative phage proteins has any homologies to genes or proteins of *Listeria* or any other bacteria which are known or suspected to be toxins, pathogenicity factors, antibiotic resistance determinants, or any known allergens. Next, a repeated dose oral toxicity study in rats was conducted, which did not produce any abnormal histological changes, morbidity or mortality. Therefore, no indications for any potential risk associated with using P100 as a food additive were found. As proof of concept, and to determine the parameters for application of P100 to foods sensitive to *Listeria* contamination, surface-ripened red-smear soft cheese was produced. Cheeses were contaminated with low concentrations of *L. monocytogenes* at the beginning of the ripening period, and P100 was applied to the surface during the rind washings. Depending on the time points, frequency and dose of phage applications, we were able to obtain a significant reduction (at least 3.5 logs) or a complete eradication of *Listeria* viable counts, respectively. We found no evidence for phage resistance in the *Listeria* isolates recovered from samples. Taken together, our results indicate that P100 can provide an effective and safe measure for the control of *Listeria* contamination in foods and production equipment.


*Keywords:* Bacteriophage; *Listeria monocytogenes*; Toxicity; Genome sequence; Food safety

## 1. Introduction

Listeriosis is an infection resulting from the ingestion of foods contaminated by *Listeria monocytogenes*, and is characterized by a variety of symptoms, from diarrhea to abortion and infections of the brain and central nervous system. Because of its high mortality rate of approximately 25–30% (Vazquez-Boland et al., 2001), the disease ranks among the most severe food-borne illnesses. It was estimated that approximately 2000 hospitalizations and 500 deaths occur annually in the United States alone, as a result of the consumption of foods contaminated with *L. monocytogenes* (Mead et al., 1999). *Listeria* does not belong to the normal flora of healthy animals or man, but is an environmental bacterium and usually contaminates foods during fermentation, processing, storage, or even packaging of foods. This includes most ready-to-eat products such as milk and cheeses (mostly soft cheese), cold cuts (different types of

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meats), hot dogs, smoked fish, seafoods, and various delicatessen items.

The currently available methods and procedures are insufficient to achieve full control of this organism, whether in the food itself, or in the food production and processing equipment and related environments. Thus, there is a need for better methods to prevent contamination, and promising novel approaches should be considered and evaluated.

Bacteriophages can be regarded as natural enemies of bacteria, and therefore are logical candidates to evaluate as agents for the control of foodborne bacterial pathogens, such as *Listeria*. The attributes of phages include the following: (i) they are designed to kill live bacterial target cells, (ii) they generally do not cross species or genus boundaries, and will therefore not affect (a) desired bacteria in foods (e.g., starter cultures), (b) commensals in the gastrointestinal tract, or (c) accompanying bacterial flora in the environment. Moreover, (iii) since phages are generally composed entirely of proteins and nucleic acids, their eventual breakdown products consist exclusively of amino acids and nucleic acids. Thus, they are not xenobiotics, and, unlike antibiotics and antiseptic agents, their introduction into and distribution within a given environment may be seen as a natural process. With respect to their potential application for the biocontrol of undesired pathogens in foods, feeds, and related environments, it should be considered that phages are the most abundant self-replicating units in our environment, and are present in significant numbers in water and foods of various origins, in particular fermented foods (reviewed by Sulakvelidze and Barrow, 2005). On fresh and processed meat and meat products, more than $10^8$ viable phage per gram are often present (Kennedy and Bitton, 1987). It is a fact that phages are routinely consumed with our food, in quite significant numbers. Moreover, phages are also normal commensals of humans and animals, and are especially abundant in the gastrointestinal tract (Furuse, 1987; Breitbart et al., 2003).

Because of their inherent specificity, phages harbor the potential for precise targeting of a bacterial contamination, without compromising the viability of other microorganisms in the habitat. A number of recent reviews (Greer, 2005; Hudson et al., 2005; Sulakvelidze and Barrow, 2005; Withey et al., 2004) summarize the current status of using phage for the control of undesired bacteria in systems other than therapy of disease in humans and animals. The potential of phages for controlling foodborne pathogens is reflected in recent studies dealing with *Salmonella* (Goode et al., 2003;Leverentz et al., 2001; Whichard et al., 2003), *Campylobacter* (Atterbury et al., 2003; Goode et al., 2003), *E. coli* (Huff et al., 2005; Toro et al., 2005), and *L. monocytogenes* (Dykes and Moorhead, 2002; Leverentz et al., 2003, 2004). However, most of the phage-host systems are highly specific, which is a general limitation of using a limited number of characterized phages to attack an unknown diversity of a given target bacterium. Solutions to circumvent this problem can include (i) careful selection and pooling of different phages with different lysis ranges, and/or (ii) the use of single broad host range phages which are able to infect all (or a majority of) the targeted organisms. The latter possibility seems much more attractive: it permits a precise definition of the agent, and use of a single phage (rather than a pooled mixture) can be expected to facilitate the process of obtaining regulatory approval.

Almost all of the phages infecting organisms of the genus *Listeria* are temperate and feature a very narrow host range (Loessner and Rees, 2005). With respect to the purpose of this study, P100 was selected because it represents one of the few known virulent phages for this genus, which are strictly lytic and therefore invariably lethal to a bacterial cell once an infection has been established. Moreover, P100 features an unusually broad host range within the genus *Listeria*, similar to phage A511 (Loessner, 1991; Loessner and Busse, 1990; van der Mee-Marquet et al., 1997). More than 95% of approximately 250 different foodborne *Listeria* isolates belonging to serovar groups 1/2, 4 (*L. monocytogenes*), and 5 (*L. ivanovii*) were infected and killed by P100 (M.J. Loessner; unpublished data).

The aim of our current study was to provide a detailed characterization of the information encoded in the phage P100 genome, perform a toxicity study with respect to the potential use of P100 as a biopreservation food additive, and show its usefulness for the control of *Listeria* in a model food system. Towards this end, we here (i) report the complete genome sequence of P100 including an in-depth bioinformatic analysis which suggests that none of the predicted proteins presents a potential health risk; (ii) show the results of an oral toxicity study in rats which indicates that there is no risk associated with P100 used as a food additive, and (iii), as a proof of concept, demonstrate the successful application of P100 for the control of *L. monocytogenes* in artificially contaminated soft cheese.

## 2. Materials and methods

### 2.1. Preparation, sequencing and bioinformatic analyses of the P100 genome

Phage P100 was first isolated eight years ago, from a sewage effluent sample taken from a dairy plant in southern Germany (M.J. Loessner; unpublished results). Liquid samples were centrifuged, filter-sterilized, and tested for presence of *Listeria* phages by spotting small drops on preformed lawns of a selection of different *Listeria* indicator strains as previously described (Loessner and Busse, 1990). One particular phage which formed large, clear plaques on most tested strains was isolated, purified, and designated as P100. A stock lysate of P100, containing approximately $3 \times 10^9$ pfu/ml (plaque forming units), was then prepared using *L. monocytogenes* WSLC 1001 as a host, and stored at 4 °C.

Propagation of P100 was performed using either *L. monocytogenes* WSLC 1001 or the non-pathogenic host *L. innocua* WSLC 2096 or WSLC 2321. Purification of

virions by polyethylene-glycol precipitation and CsCl density-gradient centrifugation, and extraction of the DNA molecules was performed as previously described (Loessner et al., 1994; Loessner and Scherer, 1995). The sequence of the P100 double-stranded DNA genome was determined using a "shotgun" cloning strategy (Loessner et al., 2000; Zimmer et al., 2003), with some modifications. In brief, approximately 10 μg purified DNA was disrupted into fragments of 0.5–5 kb size by mechanical shearing. Fragments of the desired size (1–2 kb) were inserted into a standard plasmid vector (pBluescript or pGEM), and cloned into *E. coli* XL1-Blue. Nucleotide sequencing of a total of approximately 700 inserts was performed using dye-labeled oligonucleotide primers complementary to vector sequences flanking the inserts (forward and reverse), in an automated nucleotide sequencer (ABI 3700; Applied Biosystems). After approximately 50 contigs of various lengths could be assembled, gaps were closed by using phage DNA directly as template in the sequencing reaction, employing oligonucleotide primers complementary to the ends of the contigs (primer walking). Regions of low redundancy or showing sequence ambiguities were checked again by primer walking, or by sequencing a PCR amplification product designed to encompass the region of interest.

After the complete sequence was assembled, genome coordinates were defined: nucleotide position 1 (left end of the genome) was set directly upstream of the putative terminase subunit genes. The information encoded by the P100 genome was then analyzed using Vector NTI software (version 8; InforMax), and the annotated genome and all predicted open reading frames (ORF), gene products (gp) and secondary structures were again confirmed by visual inspection. The basic prerequisites for an ORF were the presence of one of the three potential start codons ATG, TTG or GTG, a suitable ribosomal binding site (Loessner and Scherer, 1995; Loessner et al., 2000), and a length of at least 40 encoded amino acids. Nucleotide and amino acid sequence alignment searches (BlastN, BlastX, and BlastP) using the ORFs and deduced gene products, respectively, were performed with Vector NTIs integrated BLAST engine which used the non-redundant database available through the NCBI web sites (http://www.ncbi.nlm.nih.gov/). Searches for specific protein domains and conserved motifs with known function were performed using the PFAM tools available online at http://pfam.wustl.edu/hmmsearch.shtml. Transmembrane domains were predicted by using the hidden Markov model (TMHMM); available at http://www.cbs.dtu.dk/services/TMHMM/. Helix-turn-helix-Scans (HTH) were performed using SeqWeb Version 2.1.0 (GCG package), accessed via the biocomputing services of the University of Zurich (http://www.bio.unizh.ch/bioc/). Potential tRNA genes were identified using the bioinformatics tool provided by http://www.genetics.wustl.edu/eddy/tRNAscan-SE (Lowe and Eddy, 1997). Loops and hairpins were identified using HIBIO software (Hitachi) and VectorNTI, and a preliminary graphical genetic map of P100 was constructed using VectorNTI.

To screen all 174 gene products predicted to be encoded by the P100 genome (Table 1) for possible similarities to currently known protein food allergens, another in silico analysis was performed based on local alignments to the amino acid sequences of the proteins contained in the Food Allergy Research and Resource Program (FARRP) allergen database available at http://www.allergenonline.com.

### 2.2. Repeated dose oral toxicity study in rats

This study was conducted according to the current OECD principles of good laboratory practice. A total of 10 healthy male and 10 healthy female Wistar albino rats (Ace Animals, Boyertown, USA) of about 8 weeks of age were used, with a pre-test body weight range of 202–231 g per male, and 193–214 g per female. Animals were randomly selected and assigned to two groups of five males and five females per group, and individually identified by ear tags. The rats were housed 1 per cage in stainless steel wire bottom cages, in a temperature controlled animal room, with a 12 h light/dark cycle. Fresh rodent chow diet was provided ad libitum, except for the fasting period of one day prior to sacrifice. Fresh water was available ad libitum.

As test material for the oral studies, purified and concentrated ($5 \times 10^{11}$ pfu/ml) phage P100 particles suspended in phosphate-buffered saline pH 7.3 (PBS) was used. The slightly cloudy liquid was aliquoted in five tubes containing 12 ml each, and stored at 4 °C for the duration of the experiment. The phage suspension and control liquid (PBS) were orally administered once daily, over a five-day period, using a syringe and 16 gauge ball-tipped feeding needle. Animals in group 1 were dosed with 1.0 ml of P100 phage ($5 \times 10^{11}$ phages), animals in group 2 (control group) received 1.0 ml of PBS only.

Body weights were recorded pre-test and prior to termination. The animals were observed once daily for toxicity and pharmacological effects, and twice daily for morbidity and mortality. Food consumption was calculated at the end of the study. On day 8, all animals were anesthetized with ether, sacrificed, and exsanguinated.

All animals were examined for gross pathology. The esophagus, stomach, duodenum, jejunum, ileum, cecum, and colon were preserved in 10% neutral buffered formalin. Histopathologic preparation (cross-sections and longitudinal sections) and microscopical analysis were performed according to standardized procedures. All results were evaluated based on the relationship between the dose levels and incidents or severity of responses (if any). Appropriate statistical evaluations were performed using Instat Statistics Version 2.0 software.

### 2.3. Application of P100 to control Listeria on a soft cheese model

To demonstrate the usefulness of P100 for the control of *L. monocytogenes* on the surface of contaminated soft cheeses, several experiments were conducted. As a suitable

Table 1
Features of bacteriophage P100 ORFs, gene products, homologies, and functional assignments

| ORF | Start | Stop | GP (MW[a]) | GP (IP[a]) | Similarities/homologies to genes or gene products of other phages[b] | Putative functional assignments[c] |
|---|---|---|---|---|---|---|
| gp1 | 52 | 438 | 14.7 | 8.82 | — | |
| gp2 | 422 | 694 | 10.5 | 10.05 | orf118 (LP65) | |
| gp3 | 700 | 1,116 | 15.3 | 4.36 | orf34 (phage K) | |
| gp4 | 1,116 | 1,397 | 10.6 | 9.7 | orf35 (phage K); 1102phi1-3; orf115 (LP65) | |
| gp5 | 1,747 | 3,300 | 59.1 | 5.83 | orf35 (phage K); 1102phi1-3; orf115 (LP65); | Large terminase |
| gp6 | 3,369 | 4,208 | 31.5 | 5.29 | orf36 (phage K); | |
| gp7 | 4,213 | 4,410 | 7.7 | 5.32 | — | |
| gp8 | 4,400 | 5,038 | 24.4 | 4.78 | orf1 (A511) (100%); orf36 (phage K); | |
| gp9 | 5,028 | 5,408 | 14.4 | 6.94 | orf2 (A511) | |
| gp10 | 5,472 | 6,497 | 36.4 | 9.81 | ply (A511); and endolysins from other phages | Endolysin (amidase) |
| gp11 | 6,670 | 7,398 | 26.1 | 8.99 | | |
| gp12 | 7,500 | 7,820 | 12.2 | 9.82 | — | |
| gp13 | 7,822 | 8,172 | 13.6 | 5.47 | orf40 (phage K) | |
| gp14 | 8,189 | 9,832 | 61.1 | 6.39 | orf41 (phage K); orf112 (LP65); other phages | Putative portal protein |
| gp15 | 9,931 | 10,725 | 29.7 | 5.1 | orf1 (A511), orf42 (phage K), orf111 (LP65) | |
| gp16 | 10,718 | 11,620 | 33.8 | 4.47 | orf2 (A511); orf43 (phage K), | |
| gp17 | 11,790 | 13,196 | 51.5 | 5.27 | cps (A511); cps (Twort), cps (phage K); orf109 (LP65) | Major capsid protein |
| gp18 | 13,278 | 13,613 | 12.9 | 8.95 | — | |
| gp19 | 13,620 | 14,501 | 33.2 | 4.99 | orf3 (A511); orf45 (phage K); | |
| gp20 | 14,519 | 15,337 | 31.2 | 6.51 | orf4 (A511); orf46 (phage K); orf107 (LP65) | |
| gp21 | 15,337 | 15,954 | 23.9 | 10.28 | orf5 (A511); orf47 (phage K); orf106 (LP65) | |
| gp22 | 15,967 | 16,806 | 31.5 | 4.68 | orf6 (A511); orf48 (phage K); orf105 (LP65) | |
| gp23 | 16,806 | 17,126 | 12.3 | 8.69 | orf7 (A511) | |
| gp24 | 17,130 | 18,818 | 61.3 | 4.85 | Tsh (A511), orf49 (phage K); orf103 (LP65); Twort | Tail sheath protein |
| gp25 | 18,937 | 19,308 | 13.7 | 5.91 | orf8 (A511); orf50 (phage K); orf102 (LP65) | |
| gp26 | 19,459 | 19,902 | 17.3 | 4.86 | orf9 (A511); orf52 (phage K); orf100 (LP65) | |
| gp27 | 19,970 | 20,557 | 23.1 | 4.14 | orf54 (phage K); | |
| gp28 | 20,619 | 24,344 | 131 | 9.06 | orf55 (phage K); orf 98 (LP65) | |
| gp29 | 24,393 | 26,780 | 88.4 | 5.15 | orf56/57 (phage K); orf134 (LP65) | |
| gp30 | 26,798 | 28,330 | 56.8 | 4.8 | orf58 (phage K); orf97 (LP65) | |
| gp31 | 28,368 | 29,081 | 25.7 | 5.21 | orf59 (phage K); orf129/130 (LP65) | |
| gp32 | 29,086 | 29,619 | 20.2 | 5.07 | orf60 phage K; orf131 (LP65) | |
| gp33 | 29,606 | 30,316 | 26.3 | 4.74 | orf61 (phage K); orf132 (LP65) | Putative baseplate protein |
| gp34 | 30,330 | 31,376 | 39.2 | 5.02 | orf62 (phage K); orf95 (LP65) | Tail protein |
| gp35 | 31,412 | 35,341 | 146 | 4.84 | orf63 (phage K); orf94 (LP65) | |
| gp36 | 35,458 | 35,979 | 19.1 | 5.91 | orf64 (phage K); orf91 (LP65) | |
| gp37 | 35,996 | 39,451 | 128.2 | 5 | orf65 (phage K); orf90 (LP65); other phages | |
| gp38 | 39,497 | 39,718 | 8.6 | 5.24 | — | |
| gp39 | 39,922 | 41,013 | 39.2 | 7.01 | gp20 (A118), (PBSX) | |
| gp40 | 41,045 | 41,455 | 15.2 | 4.5 | — | |
| gp41 | 41,452 | 41,589 | 5.3 | 5.11 | gp17 (PSA) | |
| gp42 | 41,690 | 43,435 | 66.4 | 6.45 | orf69 (phage K); orf123 (LP65); other phages | Putative helicase |
| gp43 | 43,450 | 45,090 | 62.8 | 6.45 | orf70 (phage K) | putative replicase |
| gp44 | 45,108 | 46,571 | 55.6 | 5.89 | orf71 (phage K); orf76 (LP65); other phages | Primase-helicase |
| gp45 | 46,586 | 47,638 | 39.8 | 4.92 | — | |
| gp46 | 47,733 | 48,113 | 14.5 | 9.8 | orf74 (phage K); orf70 (LP65) | Exonuclease? |
| gp47 | 48,184 | 49,617 | 53.8 | 5 | — | |
| gp48 | 49,637 | 50,227 | 22.9 | 7.02 | orf75 (phage K) | |
| gp49 | 50,227 | 51,288 | 40.4 | 4.85 | orf76 (phage K); orf68 (LP65) | Primase |
| gp50 | 51,335 | 51,982 | 23.5 | 5.76 | Proteins from several phages and bacteria | dUTPase |
| gp51 | 51,979 | 52,203 | 8.1 | 5.28 | — | |
| gp52 | 52,200 | 52,523 | 12.2 | 4.48 | — | |
| gp53 | 52,516 | 52,938 | 16.1 | 5.33 | orf77 (phage K) | |
| gp54 | 52,941 | 53,561 | 23.6 | 5.38 | orf78 (phage K), gene2 (SPO1); (D14), (T5) | |
| gp55 | 53,678 | 55,027 | 51.7 | 5.79 | | Ribonucleoside-diphosphate reductase alpha subunit |
| gp56 | 55,238 | 56,269 | 38.8 | 5.62 | | Ribonucleoside-diphosphate reductase alpha subunit |
| gp57 | 56,445 | 57,476 | 39.5 | 4.98 | | Ribonucleoside-diphosphate reductase beta subunit |

Table 1 (*continued*)

| ORF | Start | Stop | GP (MW[a]) | GP (IP[a]) | Similarities/homologies to genes or gene products of other phages[b] | Putative functional assignments[c] |
|---|---|---|---|---|---|---|
| gp58 | 57,473 | 57,925 | 17.4 | 4.45 | | |
| gp59 | 57,928 | 58,224 | 10.8 | 5.06 | | |
| gp60 | 58,248 | 58,940 | 25.7 | 6.04 | | |
| gp61 | 58,943 | 59,080 | 5 | 9.63 | | |
| gp62 | 59,083 | 60,270 | 44.3 | 7.18 | | |
| gp63 | 60,267 | 61,181 | 34.7 | 5.28 | orf110 (phage K), (Felix 01) | Ribose-phosphate pyrophosphokinase |
| gp64 | 61,192 | 62,985 | 67.9 | 5.31 | orf111 (phage K) | Nicotinamid phosphoribosyl transferase? |
| gp65 | 63,081 | 65,540 | 95 | 7.31 | orf18 (phage K); orf137 (LP65) | |
| gp66 | 65,634 | 66,422 | 30.8 | 9.47 | orf84 (phage K); | |
| gp67 | 66,415 | 66,729 | 12 | 9.27 | orf85 (phage K); | DNA binding |
| gp68 | 66,812 | 67,648 | 31.9 | 5.34 | orf86/88/90 (phage K); SPO1; orf 59 (LP65) | DNA polymerase |
| gp69 | 67,983 | 70,091 | 80.9 | 5.67 | orf86/88/90 (phage K); SPO1; orf 59 (LP65) | DNA polymerase |
| gp70 | 70,186 | 70,662 | 18.6 | 5.03 | orf91 (phage K); | |
| gp71 | 70,700 | 71,959 | 46.7 | 4.92 | orf22 phage (Twort); orf92 (phage K) | |
| gp72 | 72,029 | 73,273 | 46.1 | 7.75 | orf93 (phage K), recombinase A (LP65) | Recombinase |
| gp73 | 73,335 | 73,712 | 14.5 | 8.95 | — | |
| gp74 | 73,712 | 74,350 | 25.2 | 7.12 | orf94 (phage K); many bacterial proteins | Potential sigma factor |
| gp75 | 74,409 | 74,570 | 6.1 | 3.93 | — | |
| gp76 | 74,769 | 74,626 | 5.5 | 6.76 | — | |
| gp77 | 74,791 | 75,498 | 26.1 | 4.91 | orf95 (phage K) | |
| gp78 | 75,606 | 75,992 | 15 | 4.93 | — | |
| gp79 | 75,989 | 76,918 | 35.5 | 6.18 | — | |
| gp80 | 76,977 | 78,248 | 47.5 | 7.87 | orf98 (phage K); orf64 (LP6) | |
| gp81 | 78,268 | 78,651 | 13.9 | 9.6 | — | |
| gp82 | 78,659 | 79,204 | 20.5 | 8.68 | — | |
| gp83 | 79,259 | 79,456 | 7.1 | 8.21 | — | |
| gp84 | 79,507 | 80,214 | 27 | 9.65 | orf101 (phage K); orf45 (LP65) | |
| gp85 | 80,225 | 80,707 | 18.6 | 10.35 | orf102 (phage K); | Alanyl-tRNA synthetase? |
| gp86 | 80,767 | 81,651 | 33.2 | 5.31 | — | |
| gp87 | 81,740 | 82,177 | 16.7 | 5.5 | — | |
| gp88 | 82,183 | 82,629 | 17.4 | 4.48 | — | |
| gp89 | 82,604 | 83,434 | 32 | 5.82 | — | |
| gp90 | 83,439 | 84,455 | 38.1 | 5.26 | orf15 (phage K) | ATPase |
| gp91 | 84,442 | 85,287 | 32.5 | 8.45 | — | |
| gp92 | 85,349 | 85,816 | 17.8 | 5.08 | — | |
| gp93 | 85,849 | 86,421 | 21.4 | 9.43 | — | |
| gp94 | 86,418 | 86,996 | 21.7 | 9.89 | — | |
| gp95 | 86,989 | 87,321 | 12.6 | 9.81 | — | |
| gp96 | 87,607 | 88,335 | 27.5 | 5.32 | orf103 (phage K), orf41 (LP65) | |
| gp97 | 88,350 | 88,817 | 17.9 | 4.38 | orf104 (phage K) | |
| gp98 | 88,932 | 89,975 | 39.5 | 5.83 | orf105 (phage K) | |
| gp99 | 90,023 | 90,592 | 21.2 | 5.29 | — | |
| gp100 | 90,595 | 91,128 | 19.7 | 7.85 | — | |
| gp101 | 91,143 | 91,901 | 29.3 | 9.36 | — | |
| gp102 | 91,914 | 92,204 | 11.6 | 9.4 | — | |
| gp103 | 92,914 | 92,633 | 10.8 | 8.31 | — | |
| gp104 | 93,942 | 94,724 | 30.4 | 5.52 | — | |
| gp105 | 94,891 | 95,100 | 8.1 | 3.95 | — | |
| gp106 | 95,213 | 95,476 | 10.4 | 10.1 | — | |
| gp107 | 95,560 | 95,835 | 10.4 | 9.75 | — | |
| gp108 | 95,948 | 96,340 | 15 | 4.09 | | |
| gp109 | 97,353 | 97,607 | 9.1 | 6.16 | — | |
| gp110 | 97,604 | 97,864 | 9.6 | 4.45 | — | |
| gp111 | 97,888 | 98,073 | 7.3 | 6.76 | — | |
| gp112 | 98,092 | 98,265 | 6.2 | 10.03 | — | |
| gp113 | 98,407 | 98,682 | 10.2 | 6.78 | — | |
| gp114 | 98,696 | 98,983 | 10.9 | 4.97 | — | |
| gp115 | 99,137 | 99,409 | 10.3 | 4.37 | — | |
| gp116 | 99,726 | 99,854 | 4.8 | 9.9 | — | |
| gp117 | 100,157 | 100,561 | 15.2 | 4.79 | Sensor protein (phi13) gp37 (PSA) | |
| gp118 | 100,564 | 100,782 | 8.2 | 5.6 | — | |

(*continued on next page*)

Table 1 (*continued*)

| ORF | Start | Stop | GP (MW[a]) | GP (IP[a]) | Similarities/homologies to genes or gene products of other phages[b] | Putative functional assignments[c] |
|---|---|---|---|---|---|---|
| gp119 | 100,784 | 101,005 | 8.3 | 5.06 | — | |
| gp120 | 101,012 | 101,248 | 9.2 | 4.58 | — | |
| gp121 | 101,245 | 101,511 | 10.1 | 4.18 | — | |
| gp122 | 101,504 | 101,995 | 18.4 | 5.07 | — | |
| gp123 | 101,998 | 102,504 | 19.3 | 4.55 | — | |
| gp124 | 102,515 | 103,699 | 46.6 | 6.77 | gp52 (PSA) (EJ-1) | |
| gp125 | 103,862 | 104,287 | 16.7 | 7.88 | — | |
| gp126 | 104,305 | 104,595 | 11.2 | 3.95 | gp37 (PSA) | |
| gp127 | 104,592 | 104,777 | 7.2 | 4.75 | | |
| gp128 | 104,777 | 105,124 | 13.3 | 5.35 | — | |
| gp129 | 105,156 | 105,416 | 10.1 | 4.08 | — | |
| gp130 | 105,496 | 105,828 | 13 | 5.3 | — | |
| gp131 | 105,829 | 106,224 | 15.3 | 9.3 | — | |
| gp132 | 106,289 | 106,468 | 6.3 | 9.11 | — | |
| gp133 | 106,491 | 106,853 | 14.3 | 5.38 | | |
| gp134 | 106,853 | 107,209 | 13.8 | 5.91 | — | |
| gp135 | 108,026 | 107,253 | 30.3 | 5.59 | | |
| gp136 | 108,359 | 108,039 | 12.2 | 4.69 | orf58 (A118) | |
| gp137 | 108,660 | 108,352 | 11.8 | 6.82 | — | |
| gp138 | 109,183 | 108,674 | 20 | 9.67 | — | |
| gp139 | 109,396 | 109,205 | 7.3 | 8.22 | — | |
| gp140 | 109,686 | 109,402 | 10.5 | 4.17 | — | |
| gp141 | 110,157 | 109,876 | 10.9 | 8.8 | — | |
| gp142 | 110,441 | 110,217 | 8.8 | 8.23 | — | |
| gp143 | 110,984 | 110,442 | 21 | 6.99 | — | |
| gp144 | 111,208 | 110,981 | 9.2 | 4.24 | — | |
| gp145 | 112,464 | 111,211 | 48.2 | 5.89 | — | |
| gp146 | 112,891 | 112,466 | 16.4 | 4.79 | — | |
| gp147 | 113,444 | 112,956 | 18.9 | 9.39 | — | |
| gp148 | 114,082 | 113,450 | 23.9 | 9.53 | — | |
| gp149 | 114,282 | 114,085 | 7.8 | 5.84 | — | |
| gp150 | 114,784 | 114,272 | 19.2 | 8.35 | — | |
| gp151 | 115,481 | 114,864 | 23.6 | 6.42 | | |
| gp152 | 115,696 | 115,478 | 8.1 | 8.97 | — | |
| gp153 | 116,090 | 115,713 | 14.4 | 4.53 | orf1 (SPO1) | |
| gp154 | 116,449 | 116,093 | 13.4 | 9.18 | — | |
| gp155 | 117,468 | 116,527 | 36.2 | 5.32 | orf21 (phage K) | Ligase? |
| gp156 | 118,018 | 117,482 | 20.3 | 4.99 | — | |
| gp157 | 118,206 | 118,015 | 7.8 | 9.95 | | |
| gp158 | 118,710 | 118,207 | 19.3 | 9.05 | orf4 (phage K) | |
| gp159 | 118,981 | 118,712 | 10.4 | 9.22 | | |
| gp160 | 120,311 | 119,031 | 47.8 | 8.18 | | |
| gp161 | 120,547 | 120,344 | 7.8 | 9.23 | — | |
| gp162 | 120,971 | 120,540 | 16.8 | 5.54 | | Pyrophosphatehydrolase |
| gp163 | 121,209 | 120,985 | 8.3 | 8.99 | — | |
| gp164 | 121,465 | 121,223 | 9.5 | 9.57 | orf36 (A118) | Repressor? |
| gp165 | 123,090 | 121,570 | 57.3 | 6.72 | (KVP40) (Aeh1) (Felix 01) | |
| gp166 | 124,019 | 123,801 | 8.2 | 9.62 | — | |
| gp167 | 125,497 | 125,090 | 16.5 | 10.06 | — | |
| gp168 | 125,720 | 125,523 | 7.7 | 6.94 | — | |
| gp169 | 128,127 | 127,855 | 10.2 | 5.8 | | |
| gp170 | 128,679 | 128,254 | 16.4 | 9.74 | — | |
| gp171 | 130,275 | 130,039 | 8.8 | 4.99 | — | |
| gp172 | 130,666 | 130,325 | 12.9 | 5.51 | — | |
| gp173 | 131,035 | 130,691 | 13.8 | 5.13 | — | |
| gp174 | 131,320 | 131,051 | 9.9 | 5.98 | — | |
| tRNA-Met | 123,714 | 123,784 | — | — | Anticodon CAT | tRNA-Met |
| tRNA-Pro | 124,678 | 124,752 | — | — | Anticodon TGG | tRNA-Pro |
| tRNA-Arg | 125,870 | 125,940 | — | — | Anticodon TCT | tRNA-Arg |
| tRNA-Gly | 126,187 | 126,257 | — | — | Anticodon TCC | tRNA-Gly |
| tRNA-Asn | 126,327 | 126,399 | — | — | Anticodon GTT | tRNA-Asn |
| tRNA-Ser | 127,020 | 127,111 | — | — | Anticodon TGA | tRNA-Ser |
| tRNA-Phe | 127,124 | 127,195 | — | — | Anticodon GAA | tRNA-Phe |

Table 1 (*continued*)

| ORF | Start | Stop | GP (MW[a]) | GP (IP[a]) | Similarities/homologies to genes or gene products of other phages[b] | Putative functional assignments[c] |
|---|---|---|---|---|---|---|
| tRNA-Lys | 127,201 | 127,272 | — | — | Anticodon TTT | tRNA-Lys |
| tRNA-Tyr | 127,280 | 127,351 | — | — | Anticodon ATA | tRNA-Tyr |
| tRNA-Trp | 127,398 | 127,469 | — | — | Anticodon CCA | tRNA-Trp |
| tRNA-Gln | 127,473 | 127,544 | — | — | Anticodon TTG | tRNA-Gln |
| tRNA-Thr | 127,563 | 127,634 | — | — | Anticodon TGT | tRNA-Thr |
| tRNA-Tyr | 127,717 | 127,798 | — | — | Anticodon GTA | tRNA-Tyr |
| tRNA-Leu | 128,160 | 128,242 | — | — | Anticodon TAG | tRNA-Leu |
| tRNA-Asp | 128,710 | 128,781 | — | — | Anticodon GTC | tRNA-Asp |
| tRNA-Ile | 128,886 | 128,957 | — | — | Anticodon GAT | tRNA-Ile |
| tRNA-Ser | 129,134 | 129,220 | — | — | Anticodon GCT | tRNA-Ser |
| tRNA-Cys | 129,302 | 129,372 | — | — | Anticodon GCA | tRNA-Cys |

[a] Predicted by computer analysis.
[b] Only the most significant homologies are listed. Names of phages are in brackets; individual references are not listed.
[c] Based upon homologies to other proteins.

test organism, *L. monocytogenes* strain LmC (serovar 1/2c) was used, originally isolated from a dairy plant known to have a persistent *Listeria* contamination in the production equipment (de Meester; unpublished). The organism was cultivated on BHI agar (Oxoid, UK) at 30 °C, and plates stored at 4 °C. P100 lysates were purified by tangential-flow ultrafiltration (30 kDa cut-off), and adjusted to approximately $1 \times 10^{10}$ pfu/ml, in MOPS buffer (10 mM 3-(*N*-morpholino) propanesulfonic acid, pH 7.3).

In preliminary experiments, an artificial cheese surface model (Ch-easy plates; NIZO) was employed to define the most suitable conditions for application of phage during ripening of cheese. Experimental modifications included (a) spiking the unripened cheese surface with *Listeria* cells at concentrations of 1 or 10 cfu/g of cheese, respectively, and (b) addition of phage P100 at various intervals to the salt brine wash (15–20% NaCl, dissolved in water), resulting in different concentrations of phage on the cheese surface.

Based upon these optimization trials (results not shown), P100 was then used during production/ripening of artificially contaminated surface ripened red-smear soft cheese (type "Munster"). The entire process was designed to simulate a commercial production process, and carried out in a fully equipped cheese-making pilot plant. Cheeses were made according to standard protocols, from pasteurized cow's milk, using a mesophilic starter culture and calf rennet. The acidified, clotted curd was cut, pressed in plastic cheese moulds, and treated in a brine bath (1.9 M NaCl) for several hours (day 0). The unripened cheeses (45% fat in dry matter, weight approx. 180 g, single flat side surface approx. 65 $cm^2$) were then surface-dried for approximately 20 h at controlled humidity. In all experimental setups, round flat cheese rinds (65 $cm^2$, corresponding to approximately 30–40 g) were then removed with sterile knifes, and placed in large plastic petri dishes, rind-side up. The rinds were then smeared at days 1, 2, 3, 4, 6, 10, 13 with 210 μl of a smearing solution consisting of 1.9 M NaCl and a mixed surface ripening flora (*Brevibacterium linens* ($10^8$ cfu/ml) and *Debaryomyces hansenii* ($10^8$ cfu/ml) (the yeast was used on day 1 only). To achieve even distribution of *Listeria* cells, they were added to the first washing solution ($6 \times 10^3$ cfu/ml), which resulted in a fairly consistent contamination density of approximately $2 \times 10^1$ cfu/$cm^2$. During ripening, cheeses were incubated at controlled temperature of 14 °C and 98% relative humidity. On day 16, cheese were packaged in parchment composite paper, and stored at 6° C until the end of the experiment.

In a first set of experiments designed to evaluate the required concentration of P100, the phage was repeatedly applied to the cheese surface. Two different concentrations were used, a higher dose ($3 \times 10^9$ pfu/ml, resulting in phage titers on the cheese surface of approximately $6 \times 10^7$ pfu/$cm^2$), and a lower dose ($1.5 \times 10^8$ pfu/ml, corresponding to approximately $2 \times 10^6$ pfu/$cm^2$ on the surface). Phage was added to all washing/smearing solutions. In a second cheese-ripening experiment, only one single dose of phage was used ($6 \times 10^8$ pfu/ml). To optimize the distribution of phage on the uneven cheese surface, 1.0 ml of smearing solution was used per cheese surface, which resulted in a phage count of $6 \times 10^7$ pfu/$cm^2$. Control cheeses received *Listeria* cells but no phage.

For sampling, the cheese rinds (65 $cm^2$, corresponding to approximately 30–40 g) were homogenized with buffer (50 mM trisodium-citrate, pH 7.3; added to 250 ml) using a Stomacher laboratory blender. The homogenate and decimal dilutions prepared thereof were surface plated on *Listeria* selective Oxford agar plates (Oxoid), in triplicate. The plates were incubated at 37 °C for 48 h, until typical *Listeria* colonies could be enumerated and viable counts calculated. The lower limit of detection was approximately 5 cfu/$cm^2$ of cheese.

To determine the possible development of resistance against P100, more than 30 of the *Listeria* colonies isolated from the Ch-easy plates during preliminary setups, and from cheeses treated with lower doses of P100 were re-purified by repeated streaking on non-selective agar plates, and subsequently challenged with P100 in lysis assays (liquid culture lysis assay and/or plaque formation in double-layer agar plates).

The titer of P100 on the cheese surfaces was determined from the same homogenized samples. To avoid microbial contamination of the soft agar double layer plates, an antibiotic-resistant indicator host strain (*L. ivanovii* $Sm^r$) was used. Volumes of 0.1 ml of decimal dilutions were mixed with 0.2 ml of log-phase bacteria and 3.5 ml BHI soft agar (0.4% agar), and poured onto the surface of a BHI plate (both media contained 300 μg streptomycin/ml). Following incubation for 16–24 h at 30 °C, plaques could be counted.

## 3. Results

### 3.1. Sequencing and bioinformatics

The complete dsDNA genome sequence of P100 of 131,384 bp was assembled from a highly redundant set of 1756 single sequence reads with an average length of 800 bp, yielding a total of 1,405,715 bp (corresponding to >10-fold average coverage). The fully annotated sequence has been deposited in GenBank, under Accession No. DQ004855.

A total of 174 open reading frames were identified, predicted to encode gene products (proteins) ranging from 5 kDa (gp61) to 146 kDa (gp35) (Table 1). In addition, P100 encodes a total of 18 tRNAs, located at the right end of the genome (nucleotide position 123,714–129,372). Solely on the basis of sequence similarities, putative functional assignments could be made to 25 of the predicted products, whereas the other proteins represent new entries in the database.

The bioinformatic analyses and annotations (in particular sequence alignments and motif searches) did not reveal any similarities of P100 genes or gene products to any genes or proteins or other factors known or believed to play a direct or indirect role in the pathogenicity or virulence of *L. monocytogenes* (Vazquez-Boland et al., 2001), or of any other infectious, toxin-producing or otherwise harmful microorganism.

P100 appears to be closely related to *Listeria* phage A511. They both feature a broad (but nevertheless different) host range within the genus *Listeria*, and belong to the same morphotype family (*Myoviridae*; Zink and Loessner, 1992). The phenotypical observations correlate well with the now available genetic data, which revealed significant nucleotide sequence homologies of P100 to the A511 genome (Loessner and Scherer, 1995; Dorscht et al., submitted for publication). On an overall scale, P100 also shared some sequence similarities with other known *Myoviridae* phages infecting Gram-positive bacteria of the low G + C cluster, such as *Staphylococcus aureus* phage K (O'Flaherty et al., 2004) and *Lactobacillus plantarum* phage LP65 (Chibani-Chennoufi et al., 2004a).

Alignments of the 174 predicted P100 proteins with all proteins and polypeptides contained in the current food allergen database returned only one match: gp71, a 419 amino acid polypeptide encoded by orf71, which showed local similarity (e-value $8 \times 10^{-10}$) of short sequence stretches in its C-terminal portion to epitopes of wheat γ-gliadin. However, these similarities appear to be based upon specific local distribution of glutamine and proline residues in these proteins, and are not expected to cause immunological cross-reaction (see Section 4).

### 3.2. Repeated dose toxicology study in rats

Oral administration of a high dose of phage P100 for five consecutive days, followed by a two day recovery period in male and female Wistar albino rats, revealed no in-life effects attributable to the material. No deaths were noted during the study. Body weight changes over the 8 day period were normal; an average increase of 48 g (males) and 24 g (females) was observed, with no differences between the test group and the control group. There were no significant ($p \leqslant 0.05$) differences in mean body weight or food consumption between the groups (data not shown). There were no abnormal physical signs or behavioral changes noted in any animal at any observation time point. There were no significant test-article related changes in any of the male or female rats given P100. Necropsy results (Table 2) were normal in all animals except one of the animals of the P100 test group which showed a small red area in the mucosa at the junction of jejunum and ileum. Multiple thin sections from this area of the gastrointestinal tract were then examined, and all were within normal histological limits with no microscopic change to correlate with the gross observation.

It was concluded that the histomorphologic observations in the male and female rats of both groups of this study are typical of those which occur spontaneously in laboratory rats of this strain and age, and administration of P100 phage had no effect on the type or incidence of these findings.

Table 2
Incidence of histomorphologic observations

| Dose group | P100 | | Control | |
|---|---|---|---|---|
| Sex | M | F | M | F |
| Number of animals/group | 5 | 5 | 5 | 5 |
| Stomach | | | | |
| # examined/normal | 5/3 | 5/4 | 5/3 | 5/4 |
| — dilatation, mucosal glands[a] | 2 | 1 | 2 | 1 |
| Esophagus | | | | |
| # examined/normal | 5/5 | 5/5 | 5/5 | 5/5 |
| Duodenum | | | | |
| # examined/normal | 5/5 | 5/5 | 5/5 | 5/5 |
| Jejunum | | | | |
| # examined/normal | 5/5 | 5/5 | 5/5 | 5/5 |
| Cecum | | | | |
| # examined/normal | 5/5 | 5/4 | 5/5 | 5/5 |
| — inflammation, mucosa, chronic[a] | | 1 | | |
| Colon | | | | |
| # examined/normal | 5/5 | 5/5 | 5/5 | 5/5 |
| Ileojejunal junction[b] | | | | |
| # examined/normal | — | 1/1 | — | — |

[a] Minimal degree.
[b] Only the female rat which showed a slight red area was tested (see text).

### 3.3. Efficacy of P100 for control of L. monocytogenes on soft cheese

The results shown in Fig. 1 demonstrate the effect of P100 on *L. monocytogenes* contamination on a surface-ripened Munster-type soft cheese. The manufacturing process used was indistinguishable from that employed in commercial production of this type of cheese, including the specific parameters of inoculation with a standardized bacterial/yeast ripening flora, ripening conditions (temperature and duration), washing of the rind, and time point of packaging.

The inhibitory effects of P100 were clearly dose-dependent. In the first set of experiments (Fig. 1A), a lower concentration of $1.5 \times 10^8$ pfu/ml was repeatedly applied, which resulted in an approximately 2–3 log decrease of *Listeria* viable counts. Although this represents a massive reduction, it was not complete elimination. However, when a higher concentration of $3 \times 10^9$ phages per ml smearing solution was used, complete eradication of viable *L. monocytogenes* was observed. This result was confirmed by selective enrichment and subsequent plating of cheese samples, which were negative for *Listeria* (results not shown). In a subsequent experiment, only a single dose of phage was applied to the cheeses, shortly after contamination with *Listeria* cells. The larger volume of smearing liquid used here (1.0 ml) permitted a better distribution of phage on the surface of the cheese. This approach also resulted in complete inhibition, i.e., *Listeria* viable counts were below the limit of detection at all times following application of P100. In contrast, the untreated control cheeses supported growth of *L. monocytogenes* to titers of generally more than $10^7$ cfu/cm$^2$.

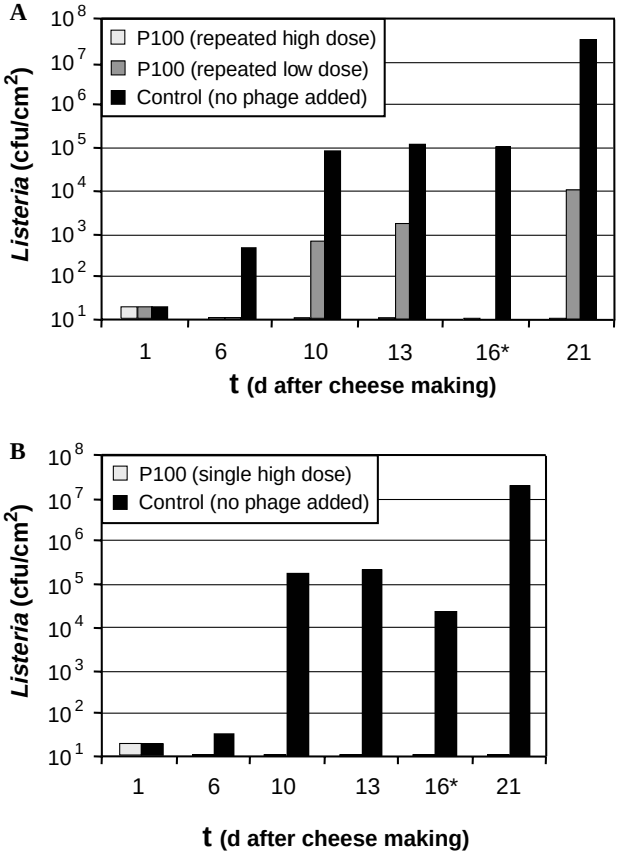


Fig. 1. Effect of phage P100 on growth of *L. monocytogenes* on surface-ripened, soft cheese with a washed rind (see text). All tested cheese were contaminated with *L. monocytogenes* on day 1 after cheese making. (A) P100 was repeatedly applied to the cheese surface at different concentrations (see text) during all rind smearings until day 13. The data point for repeated low dose application on day 16 was not measured. (B) A single high dose of P100 was added to the brine during first smearing of the cheese rind. The control cheeses received no phage. All cheeses were packaged on day 16 after cheese making (indicated by a star).

All of the *Listeria* clones re-isolated from Ch-easy plates and cheeses treated with lower concentrations of phage retained sensitivity to P100 infection, i.e., we were unable to detect development of insensitivity or resistance against the phage among the surviving *Listeria* cells. It is also important to note that Phage P100 did not noticeably affect the functioning of the natural flora and ripening process, i.e., there were no apparent changes of the P100 treated product compared to the controls, in terms of general appearance or color.

Because it was a possibility that the virions could potentially be inactivated by the proteases secreted by the microbial ripening flora, we have monitored the stability of P100 during the ripening process. However, repeated determination of phage titers recovered from the homogenized cheese surfaces before and after smearing indicated that it is sufficiently stable; no significant decrease or increase in phage titer was determined over a period of 6 days (results not shown).

## 4. Discussion

We here present a comprehensive approach to determine the suitability of P100 for the biocontrol of *L. monocytogenes*, an opportunistic foodborne pathogen causing a potentially fatal infection.

The complete genome sequence of P100 was determined and analyzed *in silico*. Bioinformatics did not indicate any similarity of any of the 174 predicted P100 gene products to any known or suspected toxins or other factors involved in regulation of virulence and/or pathogenicity of *Listeria* or other organisms. Genomic data clearly indicated that P100 is related to A511, a *Listeria* specific Myovirus whose genome has recently been sequenced (Dorscht et al., submitted for publication). Interestingly, although both phages have a very broad host range, they still show some differences in specificity, i.e., P100 is able to form plaques on some strains not infected by A511. Availability of the complete sequences together with bioinformatic analyses may permit to experimentally elucidate the molecular basis for host cell recognition and productive infection.

When the predicted gene products of P100 were aligned with proteins known or suspected to be potential food allergens, one protein (gp71) showed a local similarity in its C-terminal domain to a gamma-gliadin protein of wheat.

The e-value (probability index) calculated for each amino acid sequence alignment is supposed to indicate a possible immunological cross-reactivity. However, bioinformatic analyses also suggested that the e-value of $8 \times 10^{-10}$ was due to a spatial accumulation of glutamine (Q) and proline (P) in specific domains of these proteins. Most importantly, sequence comparisons also showed that the Q and P-rich sequences in gp71 did not match the immunoreactive epitopes of wheat gliadin (Battais et al., 2005), and there is no identical stretch of residues spanning more than 4 or 5 identical amino acids. It should also be noted that orf71 is clustered in the P100 genome with putative DNA recombination/replication elements. Therefore, gp71 is probably synthesized during the initial phase of phage infection and involved in the process of genome replication. Such proteins are not known to be components of the matured phage particle. Therefore, because of the bias in sequence alignment and based upon the predicted function of this putative protein, we conclude that gp71 has a neglectable probability to act as potential immunoreactive allergen.

In a toxicology study with rats performed under GLP criteria, a purified P100 preparation was found to be safe and well-tolerated, and no mortality, morbidity, or histopathological changes related to P100 were observed. Oral challenge studies were performed using a high dose of $5 \times 10^{11}$ phage particles given to the test animals over a period of 5 days, corresponding to approximately $2 \times 10^{12}$ phages per kilogram body weight per day. If this dose would be applied to an average human (70 kg) consuming cheese which contains P100 at a suggested concentration of $3 \times 10^{8}$ pfu/cm$^2$ and a having total rind surface of approximately 200 cm$^2$/cheese (one cheese would therefore contain a maximum of $6 \times 10^{10}$ pfu), a human would have to consume more than 2300 cheeses per day. Even if body weight difference were not considered, about 10 cheeses of approximately 180 g each per day would be required to supply the tested phage titer.

Other studies on the application of phage to animals also reported no adverse or unexpected effects of bacterial viruses on animals (Berchieri et al., 1991; Biswas et al., 2002; Cerveny et al., 2002; Chibani-Chennoufi et al., 2004b; Merril et al., 1996). In line with this, a recent a study with human volunteers receiving phage T4 indicated that it is safe for oral administration; and no phage or phage-specific antibodies could be detected in the serum of the human subjects (Bruttin and Brussow, 2005). In conclusion, there is no reason to assume that the intake of phage with food may possibly have any negative effects on humans. With respect to phage P100, the available data suggest that its use as an additive for biopreservation of foods can be expected to be safe for consumers as well as for the environment.

We have demonstrated that a preparation of *Listeria* phage P100, when applied at a suitable time point during the cheese-making and ripening process and at the proper concentration, was able to completely eradicate viable *L. monocytogenes* cells from a surface-ripened soft cheese. This compares well to other reports, where the application of a mixed preparation of different *Listeria* phages was employed to reduce contamination levels on the surface of artificially contaminated honeydew melons and apple slices (Leverentz et al., 2003). In their study, the phage mixture reduced the viable *Listeria* counts between 2.0 and 4.6 orders of magnitude on honeydew melons, whereas the effect on apples was only a 0.4 log reduction. In a follow-up study (Leverentz et al., 2004), optimized application and phage concentration enabled a reduction on honeydew melons of up to 6.8 log units after 7 days of storage. The same study also reported that higher phage concentrations more effectively reduced the pathogen contamination. The results from our study not only confirm this finding, but extend the range of foods from fruit to the more frequently contaminated milk products. Still, there is a need to further investigate the application of *Listeria* phage to be able to address the contamination problem in a wider range of foods, especially those of animal origin.

None of the *Listeria* clones isolated from cheeses receiving low concentrations of P100 revealed resistance against the phage. This was an important finding, suggesting that development of insensitivity of *Listeria* cells against strictly virulent phages such as P100, if occurring at all under these conditions, is a rare event. Clearly, such properties are crucial for preparing phage preparations and developing application protocols for the control of unwanted bacteria in any environment.

Considering the ubiquitous presence and high prevalence of phages, together with their incredible diversity and extreme specificity, it is unlikely that the addition of phages for biocontrol of specific pathogens in food would affect the consumer or the environment. Also, their application to reduce pathogens in foods can not be expected to disturb the natural microbial communities in these environments. Since phage particles constitute non-toxic, naturally present components in our foods (Kennedy and Bitton, 1987; Sulakvelidze and Barrow, 2005), they may be considered as safe for intentional application in foods. Many of the tailed phages, however, may actually not be suitable for use as natural antimicrobial, since they are temperate and can integrate their genome into the host bacterial genomes, forming a lysogen. This state in a phage life cycle is sometimes accompanied by undesired phenotypical changes, i.e., the integrated phage (prophage) can potentially carry and express genes encoding properties which increase pathogenicity and/or virulence of the host bacteria. In several cases, temperate phages have been identified as the carriers of toxins or regulators needed for development of full virulence of the host (reviewed by Boyd, 2005). This is never the case for strictly lytic (i.e., virulent) phages; they lack the genetic factors required for integration, will always enter the lytic cycle, and eventually kill and lyse the infected cells. Therefore, virulent phages seem better suited for the intended application.

It also seems preferable to select phages which are not capable of transduction, i.e., the packing of host genetic material instead of phage-encoded DNA. While many temperate *Listeria* phages were experimentally shown to be able to transduce genetic markers (Hodgson, 2000), this has not been reported for the strictly virulent phages. Some bacterial viruses even break down the bacterial DNA to generate the building blocks required for synthesis of progeny DNA. The genomes of such phages usually feature specific gene products involved in nucleotide metabolism, such as the putative ribonuleotide reductase subunits, required for conversion of ribonucleosides into desoxyribonucleosides (see P100 gp 55–57). This appears to be another desirable property of phages to be used against pathogens in food or therapy.

The first published report on use of *Listeria* phages for biocontrol used three different temperate phages from the Siphoviridae family (Roy et al., 1993). In the other previous studies dealing with *Listeria* phages and food (Dykes and Moorhead, 2002; Leverentz et al., 2003, 2004), no details of the phages were provided. However, considering the above discussed criteria for the application of phage in control of bacteria in food, feed, or medical therapy, the isolation and evaluation of phages should always be accompanied by a detailed characterization. This should encompass (i) determination of genome sequence and structure, (ii) bioinformatic analyses including all relevant databases, and, of course, (iii) proof of applicability of the phage(s) for a specified application.

The data presented here on P100 show that this phage not only has no obvious undesirable properties, but, most importantly, that it performs well when used as a natural anti-*Listeria* agent. It should also be noted that these results were obtained by using a single broad host range phage. Altogether, our results provide important data to meet the stringent requirements for obtaining approval for use in foods.

Because of the strong evidence of being effective as an agent for eradicating *L. monocytogenes* in cheese, we are currently investigating the application of this and other phages to other types of fresh foods prone to *Listeria* contamination, such as salads, hot dogs, cold cuts, seafoods, chocolate milk, and mold-ripenend soft cheese. Using phage as a natural antimicrobial may also be helpful in decontaminating food processing plants where *L. monocytogenes* is a difficult-to-eliminate part of the "house flora," whether on a steady or an intermittent basis.

In the age of genomics and genetic engineering, the defined modification of phages to further improve their antimicrobial properties also represent a possibility. An elegant approach was the construction of non-replicating killer phages (Hagens et al., 2004), which was shown to prevent cell lysis, release of intracellular components, and uncontrolled multiplication of the phage. Genetic engineering could also be helpful to change or broaden phage host ranges, and therefore enhance the currently available armamentarium for the control of pathogen contamination. However, the consumer acceptance of GMO phages in the food chain is unclear, and can be expected to prevent the application of such measures in the near to mid-term future. Because of this limitation, the isolation and characterization of naturally occurring broad host range phages such as P100 appears to be a suitable approach to harness the biological specificity of these natural enemies of bacteria.

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