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Construction of Luciferase Reporter Bacteriophage A511::*luxAB* for Rapid and Sensitive Detection of Viable *Listeria* Cells

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Specific transfer and expression of bacterial luciferase genes via bacteriophages provides an efficient way to detect and assay viable host cells. *Listeria* bacteriophage A511 is a genus-specific, virulent myovirus which infects 95% of *Listeria monocytogenes* serovar 1/2 and 4 cells. We constructed recombinant derivative A511::*luxAB*, which carries the gene for a fused *Vibrio harveyi* LuxAB protein inserted immediately downstream of the major capsid protein gene (*cps*). Efficient transcription is initiated by the powerful *cps* promoter at 15 to 20 min postinfection. Site-specific introduction of the luciferase gene into the phage genome was achieved by homologous recombination in infected cells between a plasmid carrying A511 DNA flanking *luxAB* and phage DNA. Recombinants occurred in the lysate at a frequency of 5×10^{-4} and were readily identified by the bioluminescent phenotype conferred on newly infected host cells. A511::*luxAB* can be used to directly detect *Listeria* cells. Following infection and a 2-h incubation period, numbers as low as 5×10^2 to 10^3 cells per ml were detected by using a single-tube luminometer. Extreme sensitivity was achieved by including an enrichment step prior to the *lux* phage assay; under these conditions less than 1 cell of *L. monocytogenes* Scott A per g of artificially contaminated salad was clearly identified. The assay is simple, rapid, inexpensive, and easy to perform. Our findings indicate that A511::*luxAB* is useful for routine screening of foods and environmental samples for *Listeria* cells.

Listeria monocytogenes is widely recognized as an opportunistic, food-borne pathogen. A number of major outbreaks of human listeriosis have been traced back to contaminated foods (reviewed in references 8 and 14). The standard method for detecting the genus *Listeria* (International Dairy Federation standard 143:1990) involves selective enrichment and plating on selective agar, followed by biochemical testing and species confirmation. This procedure is laborious, and results are available only after 5 to 6 days. In recent years, a number of alternative methods for more rapid detection of listerial cells have been described; these methods have been based on nucleic acid hybridization (6), nucleic acid amplification (2, 3, 29, 35, 36), or the use of specific antibodies (4, 9).

An entirely new concept for bacterial detection was introduced in 1987 by Ulitzur and Kuhn (33), who described the cloning of bacterial luciferase (*lux*) genes into the bacteriophage λ genome. Infection of host cells by the recombinant phage, phage L28, resulted in *lux* expression and bioluminescent bacteria. Reporter phages such as this one are ingenious tools for rapid detection of bacterial host cells following phage infection and a short incubation period. Numbers of cells as low as 10 *Escherichia coli* cells (33), 100 *Salmonella typhimurium* cells (30), and 10 enterobacterial cells (16) can be detected. In one recent report Sarkis et al. (28) described the introduction of a firefly luciferase gene (*FFlux*) into the genome of a temperate *Mycobacterium smegmatis* phage, which then was used to detect low numbers of lysogenic cells.

A511 is the only known phage that is virulent for members of the genus *Listeria*, and this phage is unrelated to all other *Listeria* phages (17, 20, 37). This virus is strictly genus specific (18) and has a very broad host range. It adsorbs to the listerial peptidoglycan (34), and approximately 95% of *L. monocytogenes* serovar 1/2 and 4 cells are sensitive to A511 infection (18, 22). A511 is a myovirus with a relatively large genome (genome size, approximately 116 kb [20]). At 30°C, the latent period is 55 to 60 min, and infected cells continue to lyse during the following 60 to 70 min (22). Lysis is mediated by the A511-encoded endolysin, a *Listeria* cell wall-specific *N*-acetylmuramoyl-L-alanine amidase (24). We recently cloned and sequenced a substantial part of the A511 late structural genes and identified the genes encoding the major structural proteins. Transcription of the gene for the major capsid polypeptide (*cps*) is initiated 15 to 20 min postinfection from a strong and specific promoter (21).

This study was designed to investigate whether site-specific introduction of the *lux* genes into this late-gene region results in efficient luciferase expression. We found that recombinant phage A511::*luxAB* is viable and infective and mediates high-level luciferase expression in infected cells. Our data indicate that this phage is useful as an inexpensive, rapid, and simple tool for sensitive detection of viable *Listeria* cells in foods and environmental samples.

MATERIALS AND METHODS

Bacterial strains, phages, and plasmids. The organisms and vectors used for construction of A511::*luxAB* are listed in Table 1. Other strains used in some experiments were *L. monocytogenes* Scott A (serovar 4b), WSLC 1040^T (= ATCC 15313^T) (T = type strain) (serovar 1/2a), and WSLC 1066 (= SLCC 8800) (serovar 1/2b) and *Listeria seeligeri* WSLC 4007^T (= SLCC 3954^T) (serovar 1/2b). Listeriae were cultured in brain heart infusion media (Oxoid) at 30°C, and *E. coli* cells were grown in standard Luria-Bertani media (27) at 37°C. For

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TABLE 1. Phages, bacterial strains, and plasmids involved in construction of A511::*luxAB*[a]

Phage, strain, or plasmid	Genotype or relevant properties	Source or reference
Phage A511	Virulent, *Listeria* specific, wild type	18
Strains		
L. monocytogenes WSLC 1001	Wild type, serovar 1/2 c	ATCC 19112
L. ivanovii WSLC 3009	Wild type, serovar 5	SLCC 4769
E. coli DH5αMCR	K-12 derivative, general cloning host	Life Technologies
E. coli W3110	*recA*$^+$	1
Plasmids		
pBluescriptII (SK−)	General cloning vector, Ap^r	Stratagene
pCK1	Gram$^-$-Gram$^+$ shuttle vector, Cm^r Kan^r	10
pBS511-F3s	2,123-bp *Ssp*I fragment of A511 inserted into *Sma*I site of pBluescriptII	This study
pBS511-F3s-*luxAB*	2.2-kb PCR end-modified *luxAB* fusion cloned into *Sna*BI site of pBS511-F3s	This study
pCK511-F3s-*luxAB*	4.4-kb F3s-*luxAB* cassette removed with *Bam*HI and *Eco*RI from pBS511-F3s-*luxAB* inserted into *Bam*HI-*Eco*RI-digested pCK1	This study

[a] WSLC, Weihenstephan *Listeria* Collection, Freising, Germany; ATCC, American Type Culture Collection, Rockville, Md.; SLCC, Special *Listeria* Culture Collection, Würzburg, Germany.

selection of plasmid-bearing cells, ampicillin or chloramphenicol was added at concentrations of 100 and 7 μg/ml, respectively.

Amplification of *Vibrio harveyi luxAB* gene fusion. A unique *Sna*BI site is located 50 nucleotides upstream of the 3′ end of the *cps* gene (21). The *luxAB* 5′ end PCR primer (104-mer) contained 50 nucleotides from the *Sna*BI site to the *cps* stop codon (nucleotides that are underlined), followed by a strong ribosome binding site sequence with perfect complementarity to the 3′ end of *L. monocytogenes* 16S rRNA (6) (nucleotides in boldface type), an appropriate spacer (containing only A and T) (21), and the first 38 nucleotides of *V. harveyi luxA* (nucleotides in italics) (5) in a different reading frame relative to *cps*, as follows: 5′-GTATTAGAAACGTTAAATATATTCCTGTAAAAAACGTTCATAGCAACTAAG**AGGAGGT**AAATATAT*ATGAAATTTGGAAACTTCCTTCTCACTTATCAGCCACC*-3′. The 3′ end primer (39-mer) included a *Spe*I site, a stop codon, and 24 nucleotides complementary to the 3′ end of the *V. harveyi luxB* gene (nucleotides in italics) (15), as follows: 5′-TTAATACTAGTG*TTACGAGTGGTATTTGACGATGTTGGC*-3′. A *V. harveyi luxA* and *luxB* gene fusion (*luxAB*) (12) on luciferase expression plasmid pSB322 (11) served as a template for PCR amplification with *Taq* polymerase (Boehringer). For a higher level of blunt-ended molecules, primary PCR products were end polished with *Pfu* polymerase by following the instructions of the supplier (Stratagene).

Construction of pCK511-F3s-*luxAB*. The protocol used to construct plasmid pCK511-F3s-*luxAB* is outlined in Fig. 1. A 2,123-bp *Ssp*I fragment was selected from the previously sequenced 10.15-kb A511 late gene region (21) and isolated by standard procedures (27). Ligation of the modified *luxAB* gene into the central *Sna*BI site of F3s (in pBS511-F3s) resulted in pBS511-F3s-*luxAB*. Colonies of *E. coli* DH5αMCR carrying plasmids with functional luciferase genes were identified by their bioluminescent phenotype when they were exposed to nonanal vapor (10 μl of nonanal [Aldrich] in a petri dish lid). The correct orientation and integrity of the *cps* 3′ end were checked by performing restriction endonuclease digestion and determining the nucleotide sequences of the respective regions in plasmid mini-preparations. Removal of the F3s-*luxAB* cassette with *Eco*RI and *Bam*HI, insertion into *Eco*RI-*Bam*HI-digested pCK1, and transformation into *E. coli* W3110 yielded pCK511-F3s-*luxAB*. Again, positive clones were identified by their bioluminescent phenotype, which was clearly visible with the naked eye. The plasmid was purified from *E. coli* and checked for correct structure and insertion orientation.

Homologous recombination. Two host strains for A511 (*L. monocytogenes* WSLC 1001 and *Listeria ivanovii* WSLC 3009) were transformed with pCK511-F3s-*luxAB* by electroporation of penicillin-treated cells (26). Plasmids were recovered from bioluminescent clones as previously described (23), and the integrity of plasmids was verified by restriction enzyme digestion. To allow multiple rounds of phage propagation, strains WSLC 1001(pCK511-F3s-*luxAB*) and WSLC 3009(pCK511-F3s-*luxAB*) were infected with A511 (wild type) by using standard soft-agar overlay plates (18) supplemented with 7 μg of chloramphenicol per ml. After overnight incubation at 30°C, phage from plates showing confluent lysis were eluted with 10 ml of SM buffer (27). Supernatants were filter sterilized and heat treated at 37°C for 30 min to inactivate the residual luciferase, and the titer of the phage was determined.

Identification of *lux* recombinant phages. (i) Luciferase assay. Lysates containing luciferase-expressing viruses were assayed as follows. A 900-μl portion of log-phase host bacteria (strain WSLC 3009; approximately 10^8 CFU/ml) was transferred to a polystyrene tube (75 by 12 mm; Sarstedt), mixed with 100 μl of phage lysate, and incubated at room temperature for 90 to 120 min. Samples were then assayed for bioluminescence with a tube luminometer (model Lumat LB 9501/16; Berthold). Following injection of 50 μl of 0.25% nonanal in 70% ethanol, the photons emitted were counted over a 10-s period, the values were integrated, and the results were expressed in relative light units (RLU). Negative controls (no phage added) yielded an average of 80 to 100 RLU/10 s.

(ii) Enrichment and purification of A511::*luxAB*. The primary lysate obtained from WSLC 3009(pCK511-F3s-*luxAB*) was diluted to a concentration of 10^8 PFU/ml and mixed with an equal volume (10 ml) of log-phase host cells (concentration, 5×10^8 CFU/ml). The viruses were allowed to adsorb for 20 min at 30°C. The mixture was then diluted to yield 10^5, 10^4, and 10^3 PFU/ml, and each dilution was divided into 20 1-ml portions. Following additional incubation to allow synthesis of luciferase (60 to 80 min at room temperature), samples were examined for bioluminescence as described above. The phages in the highest dilution (i.e., the sample containing the lowest number of phages) that was positive were then propagated, their titers were determined, and the procedure was repeated with higher dilutions (i.e., with samples containing fewer phages). As soon as the *lux* phage was enriched to a level of approximately 10^{-2} (i.e., one recombinant per 100 phages), single plaques were picked, groups of 10 plaques were pooled, and the viruses were eluted with buffer and assayed for bioluminescence. The positive tubes should have contained 10^{-1} *lux* phage particle. In the final purification step, 8 of 100 plaques picked conferred a bioluminescent phenotype on infected *Listeria* cells, and one of these plaques (clone 17) was selected for further study.

Verification of *luxAB* insertion. Phage was propagated and viral DNA was purified as described elsewhere (20, 27). DNA was digested with *Dra*I, *Sfu*I, and *Xba*I (United States Biochemical Corp.) and was analyzed by agarose gel electrophoresis. Southern hybridizations with digoxigenin-labeled pCK1 and *luxAB* DNAs (Random-priming kit; Boehringer) were carried out as described previously (20). A511- and A511::*luxAB*-specified mRNAs were extracted from infected listerial cells, harvested, and lysed 60 min postinfection as described previously (21, 24). Northern (RNA) hybridizations of mRNAs with a digoxigenin 3′-end-labeled *cps*-specific oligonucleotide probe and digoxigenin-labeled *luxAB* were performed as described previously (21).

Preparation of *lux* phage suspensions. *L. ivanovii* WSLC 3009 was used for large-scale phage propagation since it does not give rise to mutants resistant to A511 (19). Approximately 5×10^7 CFU/ml was infected with 5×10^4 PFU/ml in a 4-liter mixture, and the preparation was incubated at 30°C until lysis occurred (approximately 8 h). Phage particles were harvested from the lysates by tangential-flow crossfiltration (100-kDa cutoff; Sartocon module; Sartorius), washed with 0.5× brain heart infusion medium to remove luciferase and phage endolysin PLY511 (24), concentrated to a volume of 400 ml, filter sterilized (pore size, 0.22 μm), and heat treated at 37°C for 30 min to inactivate the residual luciferase; then the titer was determined and the preparation was stored at 4°C.

Determination of parameters for optimal luciferase expression and measurement. (i) Kinetics of the light-emitting reaction. A511::*luxAB*-infected *Listeria* cells in 0.5× brain heart infusion medium were incubated for 2 h at 20°C and placed in the luminometer. Following injection of aldehyde substrate, the photons emitted were counted over a 30-s period. The kinetics of the reaction were determined from a histogram printout with 20 data points.

(ii) Multiplicity of infection. Portions (100 μl) of host cells (WSLC 1001, WSLC 1040^T, and WSLC 3009; 10^6 CFU/ml) were added to 1-ml phage suspensions containing 5×10^7, 10^8, 2.5×10^8, 5×10^8, 10^9, and 2.5×10^9 PFU/ml. The resulting mixtures were incubated and assayed as described above; the signal integration time was 5 s.

(iii) Luciferase expression and stability at different temperatures. A 50-ml portion of log-phase host cells (10^7 CFU/ml) was infected with A511::*luxAB* (3×10^8 PFU/ml), and the resulting preparation was divided into 1-ml samples, which were incubated at various temperatures (10, 18, 20, 24, 30, and 37°C). Luciferase synthesis was monitored by obtaining duplicate measurements every 10 min (up to 240 min) as described above. In an additional experiment, a preparation containing 3×10^8 cells per ml was infected with 3×10^9 *lux* phage, incubated at 30°C, and assayed every 5 min for a total of 25 min.

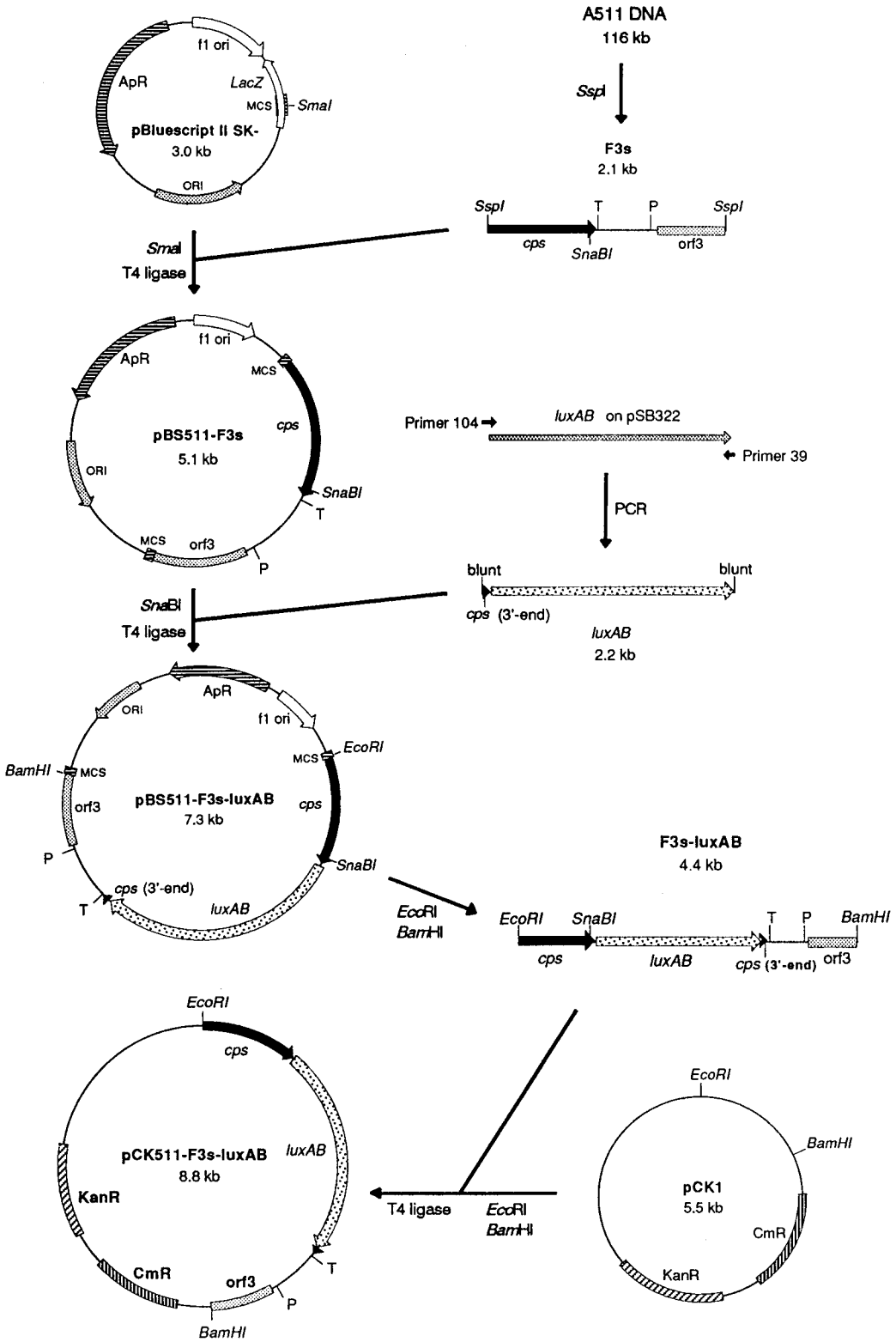

FIG. 1. Construction of *E. coli-Listeria* shuttle plasmid pCK511-F3s-*luxAB*, which carries an A511 DNA-flanked *V. harveyi luxAB* gene fusion designed for in vivo homologous recombination with phage DNA. For details, see Materials and Methods.

Detection limits. Log-phase cultures of six different strains of three listerial species (see Fig. 6) were diluted with prewarmed 0.5× brain heart infusion medium to low cell densities (10^2, 5×10^2, 10^3, 2.5×10^3, 5×10^3, and 10^4 CFU/ml), infected with 3×10^8 *lux* phage per ml, incubated for 140 min at 20°C, and assayed in duplicate for light emission (integration time, 5 s). Controls with no phage added were used to determine background light readings.

Detection of *L. monocytogenes* in artificially contaminated salad. Fresh iceberg lettuce was trimmed and cut into slices that were 1 to 2 cm wide. Portions (100

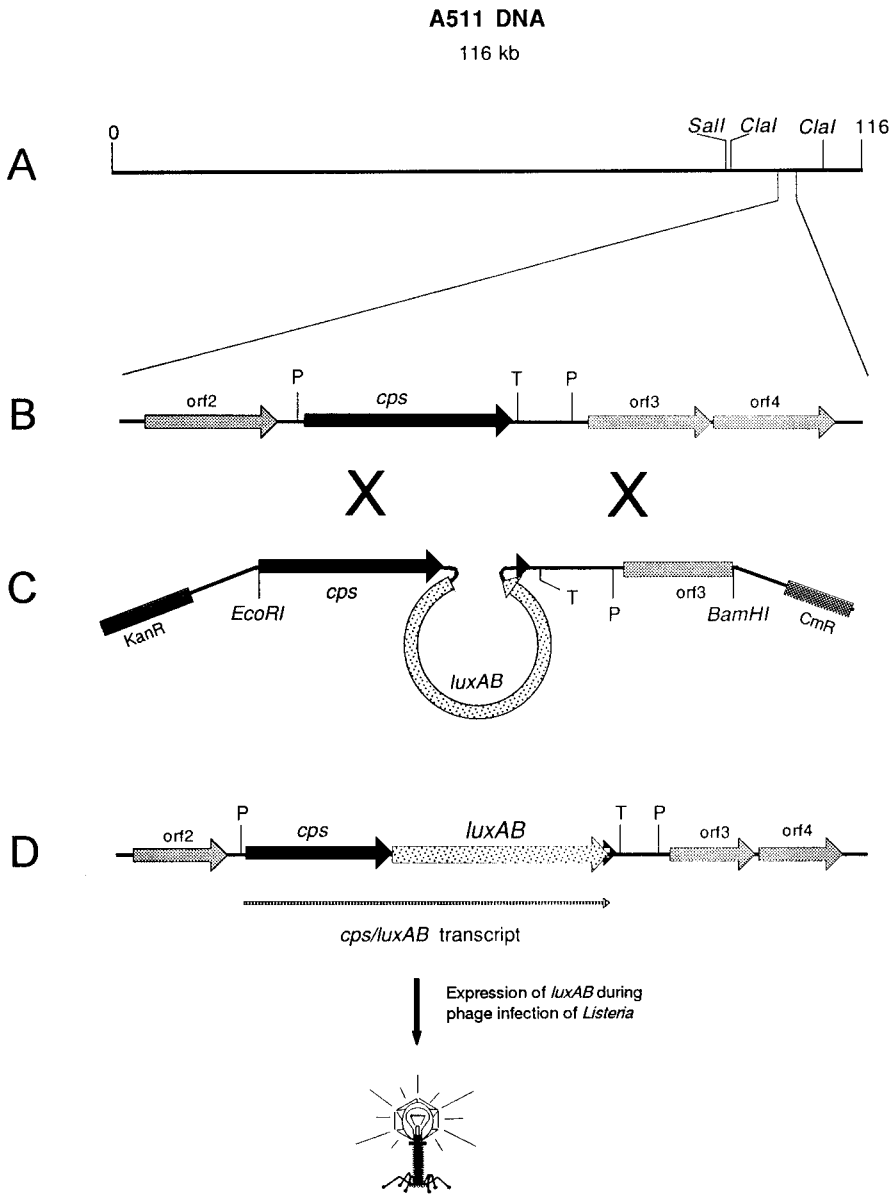

FIG. 2. Outline of the homologous recombination process leading to insertion of *luxAB* downstream of the major capsid protein gene, *cps*. (A) Simplified physical map of A511. (B) Approximately 5-kb segment of the A511 genetic map (21). The multiplication signs indicate the recombination event (double crossover) between phage DNA present in infected cells and pCK511-F3s-*luxAB* (C). (D) The resulting incorporation of *luxAB* between the 3′ end of *cps* and the downstream transcription terminator. Transcription of *cps* during late gene expression of A511::*luxAB* proceeds directly into the luciferase gene, resulting in a bicistronic *cps*/*luxAB* mRNA, which then confers a bioluminescent phenotype to infected *Listeria* cells. T, transcription terminators; P, identified promoters (21).

g) were placed in sterile polyethylene bags and spiked with 0, 0.1, 1, 10, and 100 CFU of *L. monocytogenes* Scott A per g. The bags were sealed and stored at 7°C for 1 and 7 days. A 25-g portion from each bag was mixed with 225 ml of *Listeria* selective enrichment broth (Oxoid), and were incubated at 30°C for 16 h. A 1-ml portion of each enrichment broth was then transferred to 5 ml of 0.5× brain heart infusion medium and incubated for 3 h at 30°C. Finally, duplicate 1-ml portions of each sample were mixed with 3×10^8 A511::*luxAB* particles, incubated, and assayed as described above.

RESULTS

Site-specific introduction of *luxAB* into the A511 genome. Our goal was to insert the bacterial luciferase genes directly downstream of the A511 major capsid protein gene, *cps*, without disrupting any phage-encoded genes. Transcription initiation from the strong *cps* promoter should result in high-level expression of *luxAB*. Insertion of *luxAB* into the *Sna*BI site of *cps* required regeneration of this gene during the cloning process. This was achieved by repeating the terminal 50 nucleotides of *cps* in the *luxAB* 5′ end PCR primer and ligating the blunt-ended PCR product into the *Sna*BI site of *cps*. Direct cloning into A511 phage DNA was not feasible because of its large size, the lack of appropriate, unique restriction sites, and the lack of transfection techniques for *Listeria* strains. Therefore, homologous recombination between phage DNA and a plasmid carrying *luxAB* flanked by A511 DNA was the cloning strategy which we used.

The process leading to in vivo homologous recombination is outlined schematically in Fig. 2. Subsequent analysis of the recombinant phage DNA and mRNA (Fig. 3) confirmed that

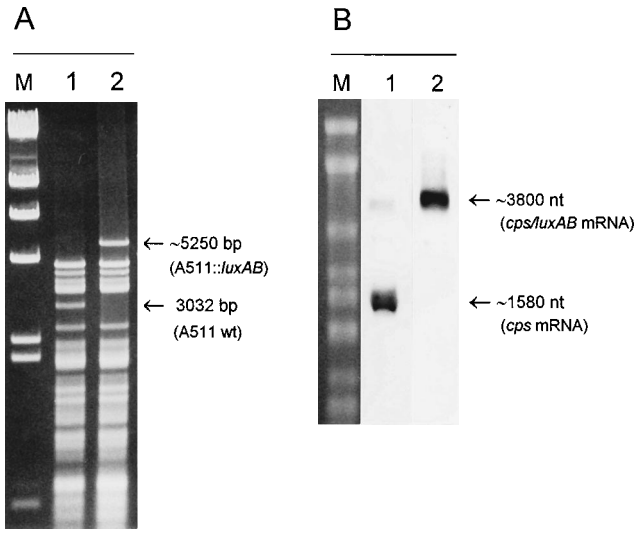

FIG. 3. (A) Separation of *Dra*I-digested phage DNA fragments by agarose gel electrophoresis. The insertion of *luxAB* into a 3,032-bp *Dra*I fragment of wild-type A511 (lane 1) resulted in an ~5,250-bp fragment in A511::*luxAB* (lane 2). Lane M contained a molecular size marker (*Hin*dIII-digested phage λ DNA). (B) Northern hybridization showing the sizes of mRNAs transcribed from the wild-type A511 *cps* gene (lane 1) and the *cps*/*luxAB* operon in A511::*luxAB* (lane 2). Lane M contained an RNA size standard mixture (7.4, 5.3, 2.8, 1.9, 1.6, 1.0, 0.6, and 0.4 kb). wt, wild type; nt, nucleotides.

the recombination event was based on a double crossover, since the sizes of the restriction fragments (data for *Sfu*I and *Xba*I are not shown) and the *cps*-specific mRNA increased by exactly the length of the fused *luxAB* gene introduced downstream of *cps*. All other DNA fragments remained unchanged, and deletions were not observed. Also, the plasmid DNA was not integrated into the luciferase-expressing phages, as shown by the lack of hybridization to labeled pCK1 DNA (data not shown). Integration of the 2.2-kb fragment into the A511 chromosome had no obvious effect on phage viability or other phenotypic characteristics.

Identification and purification of A511::*luxAB*. Expression of luciferase is an ideal marker which obviates the need for DNA-DNA hybridization to identify recombinant phage. Our purification strategy was designed to select for functional genes and phages and involved expression screening with subsequent enrichment of samples that gave the highest light output. Eventually, viruses from single plaques were directly used for infection, which resulted in purification of A511::*luxAB*. The number of *luxAB*-expressing phage in the primary lysates reflected an unexpected high frequency of homologous recombination (approximately 5×10^{-4}; i.e., one *lux* phage per 50,000 viable A511 wild-type particles).

Kinetics of the light-emitting reaction. Bacterial luciferase (EC 1.14.14.3) catalyzes the intracellular oxidation of a reduced hydrogen carrier (reduced flavin mononucleotide [$FMNH_2$]) and a long-chain aliphatic aldehyde (such as nonanal) by molecular oxygen (25, 31). Light emission (at 495 nm) depends on the ready availability of the three substrates. The kinetics determined in this study showed that peak luminescence occurred immediately after aldehyde injection and that there was a subsequent decrease to approximately 15% intensity after 30 s. Therefore, a short measurement and signal integration time yielded the best signal-to-background ratio; 5 s was used in all subsequent experiments.

Assay efficiency is dependent on phage concentration (i.e., multiplicity of infection). We performed an experiment with relatively low numbers of listerial cells (10^5 CFU/ml) to simulate conditions where A511::*luxAB* would be used for detection purposes. In general, 10^8 to 5×10^8 PFU/ml yielded the highest levels of luminescence with the three strains tested. Interestingly, 10^9 or more phage per ml decreased the efficiency of the assay significantly. A concentration of 3×10^8 PFU/ml was used as the standard concentration in most of the subsequent experiments.

Effect of temperature on synthesis and stability of LuxAB. As shown in Fig. 4, the incubation temperature had a substantial influence on levels of luciferase activity. At 10°C, newly synthesized luciferase is probably very stable. However, at this temperature the metabolism of infected host cells was apparently slowed down to a level which did not permit a normal time course of phage gene expression. Incubation at 20°C led to the highest levels of bioluminescence, and maximum bioluminescence occurred at 140 to 180 min postinfection. Considerably lower readings were obtained following incubation at 30°C: there was almost constant light emission from 100 to 240 min postinfection. This might have been due to a balance between synthesis and inactivation of the LuxAB enzyme at this temperature. Incubation at 37°C almost completely suppressed the luciferase reaction, since the fusion protein is unstable at this temperature (7, 11, 12).

Expression of phage-encoded luciferase in different *Listeria* strains. The levels of luciferase synthesis (i.e., light output) in different hosts that allow multiplication of A511::*luxAB* were found to be slightly different (Fig. 5A). For example, the light output from infected *L. monocytogenes* WSLC 1001 cells was lower than that from the other two strains tested. Using higher concentrations of cells and phages, we determined the starting point of luciferase synthesis following phage infection (Fig. 5B). In all three strains, luminescence was first detected at 15 min postinfection, and there was an exponential increase in light levels thereafter.

Direct detection of cells without enrichment. Figure 6 shows the results obtained when low numbers of cells of six different *Listeria* strains were directly challenged with A511::*luxAB* without a prior enrichment step. Although a weak signal was observed with 100 cells per ml, this signal could not be clearly distinguished from the background signal. However, as few as 500 to 1,000 cells gave signals that were twofold or more

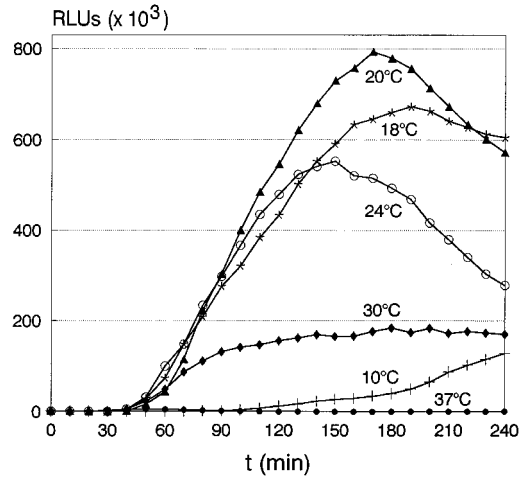

FIG. 4. Temperature-dependent enzymatic activity of LuxAB following infection of *L. ivanovii* WSLC 3009 with A511::*luxAB* and incubation at different temperatures. Samples were assayed for bioluminescence (expressed in RLU) every 10 min.

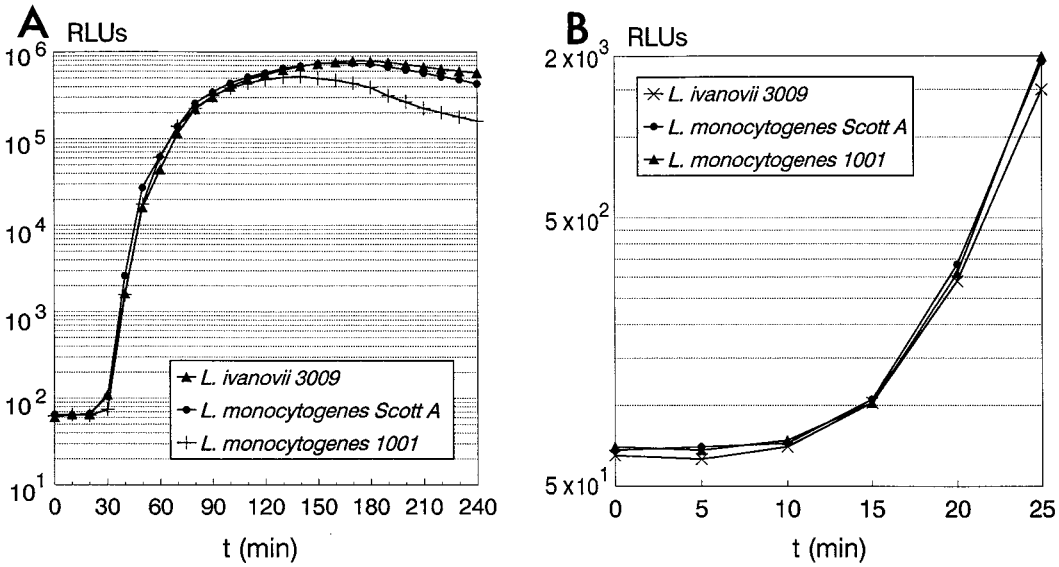

FIG. 5. Phage-induced bioluminescence in three different listerial strains. (A) Preparations containing 10^7 cells per ml were infected with 3×10^8 A511::*luxAB* particles per ml at zero time and incubated at 20°C for 240 min. Samples were assayed for bioluminescence (expressed in RLU) every 10 min. (B) Determination of the beginning of detectable luciferase synthesis in A511::*luxAB*-infected cells. Approximately 3×10^8 cells per ml were mixed with 3×10^9 recombinant *lux* phage particles and incubated at 30°C. Light emission was recorded immediately after phage was added and every 5 min thereafter up to 25 min postinfection.

greater than the background signal and therefore allowed positive identification.

Use of A511::*luxAB* as a *Listeria*-specific reporter in contaminated food. A model experiment was carried out with artificially contaminated salad in order to verify that specific enrichment prior to the *lux* phage assay can improve the detection limits to extremely low numbers of cells. Table 2 shows that the initial presence of less than one *L. monocytogenes* Scott A cell per g of salad could be unambiguously identified. The total assay time, including incubations and luciferase measurement, was 22 to 24 h. Our data also allowed a semiquantitative approximation of cell numbers; however, this was possible only when experimental conditions remained constant. The slight increase (in RLU) from day 1 to day 7 suggests that *L. monocytogenes* Scott A did not multiply significantly under the conditions used.

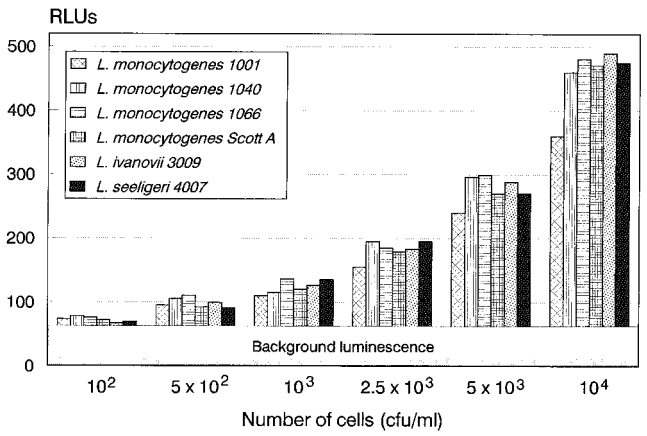

FIG. 6. Detection of low numbers of cells of various *Listeria* strains with *lux* phage. Cultures were diluted to the concentrations shown, infected with 3×10^8 A511::*luxAB* particles per ml, incubated at 20°C for 130 min, and assayed for bioluminescence. The level of background luminescence (approximately 60 RLU/5 s) is indicated at the bottom.

DISCUSSION

In this paper we describe the construction of recombinant *Listeria* bacteriophage A511::*luxAB*, in which a *V. harveyi* luciferase gene fusion was introduced into a defined site in the late genes. Transcription of *luxAB* is controlled by the powerful major capsid protein gene promoter P_{cps} and a downstream terminator, and translation is initiated by a synthetic, optimized ribosome binding site. When luciferase was used as the reporter for gene expression, bioluminescence was first detected at the same time that the *cps*-specific mRNA occurred in the cells (i.e., 15 to 20 min after injection of the phage genome) (21). This demonstrates the tight control and specificity of *cps*/*luxAB* transcription.

An important observation was that insertion of the 2.2-kb *luxAB* fragment was not accompanied by deletions in other genetic regions, as is the case with L5::*FFlux* (28). No detailed information on the physical characteristics of the A511 DNA molecule is available. However, this molecule is relatively large (116 kb) (20), and we speculate that terminal redundancy of the genome may have compensated for the relative size increase of a unit genome (plus 2.2 kb).

It is noteworthy that there was a high frequency of homologous recombination in A511-infected cells, in which approx-

TABLE 2. Assay of iceberg lettuce artificially contaminated with *L. monocytogenes* Scott A and tested with A511::*luxAB* after 1 and 7 days of storage at 7°C

Concn (cells/g)	Amt of light (RLU) after[a]:	
	1 day	7 days
0 (control)	80	95
0.1	290	410
1.0	2,600	3,400
10	29,000	32,000
100	308,000	381,000

[a] Values are means of values from two independent experiments in which duplicates were used; the standard error ranged from 3 to 10%.

imately 1 in 50,000 progeny phage integrated the *luxAB* gene by double crossover. This process was possible because of the relatively large segments of A511 DNA flanking *luxAB* on pCK511-F3s-*luxAB*. However, it remains to be determined whether such efficient recombination is generally the case in the genus *Listeria*, is brought about by specific phage-encoded (recombination) genes, or is due to the rolling-circle mode of replication of shuttle vector pCK1 (10).

A511 is a virulent phage; i.e., it is lytic for all sensitive host strains. Hence, luciferase expression is transient, and light production must be measured in a specified time interval following infection. Cell lysis is usually complete after 3 h, when infected cultures clear almost completely. However, there is still considerable light output from tubes incubated for 4 h or more (Fig. 4 and 5). Although the reason for this is not entirely clear, it indicates that liberated luciferase may function in the culture medium.

The practical applications for A511::*luxAB* are numerous. Since neither the phage nor the bacteria emit light by themselves, it is possible to harness the biological specificity of this reaction to detect low numbers of *Listeria* cells even in the presence of high numbers of other microorganisms. A511 is specific for the genus *Listeria*, yet has a very broad lytic range within this genus. This leads to few false-negative responses, especially with *L. monocytogenes* serovars 1/2 and 4, which have been found to cause most, if not all, outbreaks of listeriosis (8, 14). However, members of other *Listeria* species (*L. ivanovii*, *L. seeligeri*, *L. innocua*, and *L. welshimeri*) can also be infected and permit luciferase expression, which may necessitate subsequent isolation and species differentiation. The generation (or defined engineering) of host range mutants could overcome these limitations, in particular with respect to the few strains that are insensitive to the phage.

In foods, the detection limits obtained by various molecular techniques range from less than 1 cell per 30 ml of milk (29) to more than 10^8 cells per 0.5 g of soft cheese (35). Common to these methods are their often sophisticated procedures, which may be time consuming and require dedicated execution. In this paper we describe an alternative, rapid, sensitive procedure which is easy to carry out, inexpensive, and amenable to automation. Only viable cells yield a signal, and the detection limit in the direct assay is comparable to or better than the results achieved with PCR methods or nucleic acid hybridization. Moreover, when a short enrichment period is included in the protocol prior to the *lux* phage assay, extremely low cell numbers (less than 1 CFU/g) can be detected in a relatively short time. Considering the ease of the assay, this compares favorably to other techniques. The performance of A511::*luxAB* in routine tests for *Listeria* cells in a large number of dairy products, other foods, and environmental samples is being evaluated. The results of these tests are being compared with results obtained by the standard identification method and will be reported elsewhere. Our preliminary data indicate the superior sensitivity of the new assay.

It is also possible to assess the effect of an antibiotic or sanitizer on *Listeria* cells by using luciferase-expressing *Listeria* phage. This approach has been used with *lux* phage constructions for *E. coli* (33) and *Mycobacterium tuberculosis* (13), and it allows workers to rapidly test for specific susceptibilities on the basis of impaired cellular metabolism after exposure to drugs or chemicals, which ultimately leads to decreased or abolished light emission from *lux* phage-infected cells.

Another application of the *lux* recombinant virus is quantitative determination of *Listeria* cells, which can be done in the following two ways: (i) under standardized conditions, the light output of phage-infected cells of a given strain is nearly proportional to the cell number over the range from approximately 10^3 to 10^8 CFU/ml, which permits estimation of cell numbers on the basis of RLU readings (22, 33); and (ii) a *lux* phage-based most-probable-number method (32) is presently being developed for enumeration of low numbers of *Listeria* cells (22). The latter approach could be particularly useful when performed in a microplate format in conjunction with a microplate luminometer.

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REFERENCES

1. **Bachmann, B. J.** 1972. Pedigrees of some mutant strains of *Escherichia coli* K-12. Bacteriol. Rev. **36:**525–557.
2. **Bessesen, M. T., Q. Luo, H. A. Rotbart, M. J. Blaser, and R. T. Ellison III.** 1990. Detection of *Listeria monocytogenes* by using the polymerase chain reaction. Appl. Environ. Microbiol. **56:**2930–2932.
3. **Bsat, N., and C. Batt.** 1993. A combined modified reverse dot-blot and nested PCR assay for the specific non-radioactive detection of *Listeria monocytogenes*. Mol. Cell. Probes **7:**199–207.
4. **Bubert, A., P. Schubert, S. Köhler, R. Frank, and W. Goebel.** 1994. Synthetic peptides derived from *Listeria monocytogenes* p60 protein as antigens for the generation of polyclonal antibodies specific for secreted cell-free *L. monocytogenes* p60 proteins. Appl. Environ. Microbiol. **60:**3120–3127.
5. **Cohn, D. H., A. J. Mileham, M. I. Simon, K. H. Nealson, S. K. Rausch, D. Bonam, and T. O. Baldwin.** 1985. Nucleotide sequence of the *luxA* gene of *Vibrio harveyi* and the complete amino acid sequence of the α subunit of bacterial luciferase. J. Biol. Chem. **260:**6139–6146.
6. **Emond, E., I. Fliss, and S. Pandian.** 1993. A ribosomal DNA fragment of *Listeria monocytogenes* and its use as a genus-specific probe in an aqueous-phase hybridization assay. Appl. Environ. Microbiol. **59:**2690–2697.
7. **Escher, A., D. J. O'Kane, J. Lee, and A. Szalay.** 1989. Bacterial luciferase αβ fusion protein is fully active as a monomer and highly sensitive *in vivo* to elevated temperature. Proc. Natl. Acad. Sci. USA **86:**6528–6532.
8. **Farber, J. M., and P. I. Peterkin.** 1991. *Listeria monocytogenes*, a food-borne pathogen. Microbiol. Rev. **55:**476–511.
9. **Fliss, I., M. St. Laurent, E. Emond, R. Lemieux, R. E. Simard, A. Ettriki, and S. Pandian.** 1993. Production and characterization of anti-DNA-RNA monoclonal antibodies and their application in *Listeria* detection. Appl. Environ. Microbiol. **59:**2698–2705.
10. **Gasson, M. J., and P. H. Anderson.** 1985. High copy number plasmid vectors for use in lactic streptococci. FEMS Microbiol. Lett. **30:**193–196.
11. **Hill, P. J., C. E. D. Rees, M. K. Winson, and G. S. A. B. Stewart.** 1993. The application of *lux* genes. Biotechnol. Appl. Biochem. **17:**3–14.
12. **Hill, P. J., S. Swift, and G. S. A. B. Stewart.** 1991. PCR based engineering of the *Vibrio harveyi lux* operon and the *Escherichia coli trp* operon provides for biochemically functional native and fused gene products. Mol. Gen. Genet. **226:**41–48.
13. **Jacobs, W. R., Jr., R. G. Barletta, R. Udani, J. Chan, G. Kalkut, G. Sosne, T. Kieser, G. J. Sarkis, G. F. Hatfull, and B. R. Bloom.** 1993. Rapid assessment of drug susceptibilities of *Mycobacterium tuberculosis* by means of luciferase reporter phage. Science **260:**819–822.
14. **Jay, J. M.** 1992. Foodborne listeriosis, p. 510–552. *In* Modern food microbiology, 4th ed. Van Nostrand Reinhold, New York.
15. **Johnston, T. C., R. B. Thompson, and T. O. Baldwin.** 1986. Nucleotide sequence of the *luxB* gene of *Vibrio harveyi* and the complete amino acid sequence of the β subunit of bacterial luciferase. J. Biol. Chem. **261:**4805–4811.
16. **Kodikara, C. P., H. H. Crew, and G. S. A. B. Stewart.** 1991. Near on-line detection of enteric bacteria using *lux* recombinant bacteriophage. FEMS Microbiol. Lett. **83:**261–266.
17. **Loessner, M. J.** 1991. Improved procedure for bacteriophage typing of *Listeria* strains and evaluation of new phages. Appl. Environ. Microbiol. **57:**882–884.
18. **Loessner, M. J., and M. Busse.** 1990. Bacteriophage typing of *Listeria* species. Appl. Environ. Microbiol. **56:**1912–1918.
19. **Loessner, M. J., S. Goeppl, and M. Busse.** 1991. The phagovar variability of *Listeria* strains under the influence of virulent and temperate bacteriophages. Lett. Appl. Microbiol. **12:**192–195.
20. **Loessner, M. J., I. B. Krause, T. Henle, and S. Scherer.** 1994. Structural proteins and DNA characteristics of 14 *Listeria* typing bacteriophages. J. Gen. Virol. **75:**701–710.
21. **Loessner, M. J., and S. Scherer.** 1995. Organization and transcriptional analysis of the *Listeria* phage A511 late gene region comprising the major

capsid and tail sheath protein genes *cps* and *tsh*. J. Bacteriol. **177:**6601–6609.
22. **Loessner, M. J., and S. Scherer.** Unpublished data.
23. **Loessner, M. J., A. Schneider, and S. Scherer.** 1995. A new procedure for efficient recovery of DNA, RNA, and proteins from *Listeria* cells by rapid lysis with a recombinant bacteriophage endolysin. Appl. Environ. Microbiol. **61:**1150–1152.
24. **Loessner, M. J., G. Wendlinger, and S. Scherer.** 1995. Heterogeneous endolysins in *Listeria monocytogenes* bacteriophages: a new class of enzymes and evidence for conserved holin genes within the siphoviral lysis cassettes. Mol. Microbiol. **16:**1231–1241.
25. **Meighen, E. A.** 1991. Molecular biology of bacterial bioluminescence. Microbiol. Rev. **55:**123–142.
26. **Park, S. F., and G. S. A. B. Stewart.** 1990. High efficiency transformation of *Listeria monocytogenes* by electroporation of penicillin treated cells. Gene **94:** 129–132.
27. **Sambrook, J., T. Maniatis, and E. F. Fritsch.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
28. **Sarkis, G. J., W. R. Jacobs, Jr., and G. F. Hatfull.** 1995. L5 luciferase reporter mycobacteriophages: a sensitive tool for the detection and assay of live mycobacteria. Mol. Microbiol. **15:**1055–1067.
29. **Starbuck, M. A. B., P. J. Hill, and G. S. A. B. Stewart.** 1992. Ultra sensitive detection of *Listeria monocytogenes* in milk by the polymerase chain reaction (PCR). Lett. Appl. Microbiol. **15:**248–252.
30. **Stewart, G. S. A. B., T. Smith, and S. Denyer.** 1989. Genetic engineering for bioluminescent bacteria. Food Sci. Technol. Today **3:**19–22.
31. **Stewart, G. S. A. B., and P. Williams.** 1992. *Lux* genes and the applications of bacterial bioluminescence. J. Gen. Microbiol. **138:**1289–1300.
32. **Turpin, P. E., K. A. Maycroft, J. Bedford, C. L. Rowlands, and E. M. H. Wellington.** 1993. A rapid luminescent-phage based MPN method for the enumeration of *Salmonella typhimurium* in environmental samples. Lett. Appl. Microbiol. **16:**24–27.
33. **Ulitzur, S., and J. Kuhn.** 1987. Introduction of *lux* genes into bacteria, a new approach for specific determination of bacteria and their antibiotic susceptibility, p. 463–472. *In* J. Schlomerich, R. Andreesen, A. Kapp, M. Ernst, and W. G. Woods (ed.), Bioluminescence and chemiluminescence new perspectives. John Wiley & Sons, New York.
34. **Wendlinger, G., M. J. Loessner, and S. Scherer.** Bacteriophage receptors on *Listeria monocytogenes* cells are the N-acetyl-glucosamine and rhamnose substituents of teichoic acids or the peptidoglycan. Microbiology, in press.
35. **Wernars, K., C. J. Heuvelman, T. Chakraborty, and S. H. W. Notermans.** 1991. Use of the polymerase chain reaction for direct detection of *Listeria monocytogenes* in soft cheese. J. Appl. Bacteriol. **70:**121–126.
36. **Wiedmann, M., A. Stolle, and C. Batt.** 1995. Detection of *Listeria monocytogenes* in surface swabs using a non-radioactive polymerase chain reaction-coupled ligase chain reaction assay. Food Microbiol. **12:**151–157.
37. **Zink, R., and M. J. Loessner.** 1992. Classification of virulent and temperate bacteriophages of *Listeria* spp. on the basis of morphology and protein analysis. Appl. Environ. Microbiol. **58:**296–302.