How novel methods can help discover more information about foodborne pathogens

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Considerable emphasis is being placed on quantitative risk assessment modelling as a basis for regulation of trade in food products. However, for models to be accurate, information about the behaviour of potential pathogens in foods needs to be available. The question is how to obtain this knowledge in a simple and cost effective way. One technique that has great potential is the use of reporter bacteria which have been genetically modified to express a phenotype that can be easily monitored, such as light production in luminescent organisms. Bacteria carrying these (*lux*) genes can easily be detected using simple luminometers or more sophisticated low light imaging equipment.

By monitoring light output from these bacteria over time, it can easily be determined if the organism is growing (resulting in an increase in light emission), is dead (causing a decrease in light production) or is injured (light output remains constant). The use of imaging systems allows the response of bioluminescent bacteria to be studied directly on the food, making the technique even more useful. Applications of bioluminescence are discussed below and include use as reporters of gene expression; biocide efficacy and antibiotic susceptibility; sub-lethal injury; adhesion and biofilm formation; the microbial ecology of foods; pathogenesis; and as biosensors.

Key Words: *Bioluminescence; Ecology; Foodborne pathogens; Gene expression*

Comment des méthodes novatrices peuvent permettre de découvrir plus d'informations sur les pathogènes transmis par la nourriture

RÉSUMÉ: Un accent considérable est mis sur la modélisation pour l'évaluation du risque quantitatif comme base de réglementation du commerce de produits alimentaires. Cependant, pour que ces modèles soient précis, l'information sur le comportement de pathogènes potentiels dans les aliments doit être disponible. Mais comment peut-on obtenir cette information de façon simple et rentable ? Une technique prometteuse est l'utilisation de bactéries-reporters que l'on a modifiées génétiquement pour exprimer un phénotype dont le monitorage est facile, comme la production de lumière dans les organismes luminescents. Il est facile de déceler les bactéries dotées de tels gènes (*lux*) au moyen de simples luminomètres ou d'un équipement d'imagerie de la lumière faible plus élaboré.

En surveillant la quantité de lumière produite par ces bactéries dans le temps, on peut facilement déterminer si l'organisme croît (ce qui entraîne une plus forte émission de lumière), s'il est mort (ce qui entraîne une diminution de la production de lumière) ou s'il est lésé (la production de lumière reste constante). L'utilisation d'appareils d'imagerie permet d'étudier la bioluminescence des bactéries directement sur la nourriture, ce qui renforce l'utilité de cette technique. Les applications de la bioluminescence sont discutées ci-dessous et comprennent l'utilisation de reporters de l'expression des gènes ; l'efficacité biocide et la sensibilité aux antibiotiques ; la lésion sublétale, l'adhérence et la formation du biofilm ; l'écologie microbienne des aliments ; la pathogenèse ; et les biocapteurs.

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Bioluminescence involves the emission of visible light by living organisms through a series of enzyme-catalyzed reactions. Luminescent organisms are found in several ecological niches and comprise species of bacteria, dinoflagellates, fungi, protozoa, sponges, jellyfish, squid, starfish, worms, fish and beetles (1). The biochemistry and genetics of the light reaction have been widely studied, but other potential applications of luminescence are now being explored by scientists in other disciplines. This review will explore the ways in which bioluminescence has been used to gain insights into the behaviour of foodborne pathogens.

The genes responsible for bioluminescence in a wide variety of organisms have been identified and cloned (Table 1), and the molecular biology of bacterial bioluminescence has been described in a series of reviews by Meighen (2-5), and Meighen and Dunlop (6). The relation between the genes and enzymes of the *lux* operon and the bioluminescence reaction is diagrammatically represented by Figure 1.

The *lux* genes have been transferred and expressed in several microorganisms, including foodborne pathogens (Table 2). Luminescent phenotypes have been obtained for Gram-positive and Gram-negative bacteria. However, light output obtained from Gram-positive bacteria is usually about 100 times lower than that from comparable Gram-negative genera, and this is probably the result of poor gene expression (7).

Several reviews on the applications of luminescence have been published (8-19), and many of these applications have relevance to food safety microbiologists.

REPORTER OF GENE EXPRESSION

The 'reporter' gene concept arose from an inability to easily monitor the products of many genes. A gene with a readily assayed product (the reporter) is fused with the gene of interest so that when the latter is expressed, the product of the reporter gene is synthesized and can be detected. Schauer (20) and Nordeen (21) reviewed the use of luciferase genes for visualizing gene expression, and the former stated that this not only enabled easy detection of transcription or translation, but also allowed localization of the event in cells or organelles. Using photon imaging techniques, O'Kane et al (22) showed that it was possible to detect *Rhizobium* species carrying the *luxAB* genes in single cells from infected plants, and these techniques allow gene expression to be visualized in single mammalian cells (23) and bacterial cells (24). The use of the *luxAB* gene as a molecular reporter is not without limitations, and Blouin et al (25) have cautioned against using the system without appropriate protocol design.

More recently, attention has been focused on the green fluorescent protein gene of *Aequorea victoria* as a marker for gene expression (26,27). It does not require any substrates or additional co-factors to fluoresce, but the GFP fluorescence phenotype does not indicate the metabolic status of cells. By using a dual *gfp-luxAB* marker system, it is possible to monitor cell number and metabolic activity of specific bacterial populations simultaneously (28).

Expression of toxin and virulence genes: Gene fusions of bioluminescent genes and toxin-producing or virulence

TABLE 1
Cloned luciferases and photoproteins

Organism	Cloned gene	Reference
Bacteria		
Photobacterium leiognathi	Luciferase structural genes	114
Photobacterium phosphoreum	Luciferase structural genes	115
Vibrio fischeri	Luciferase structural genes	116
Vibrio harveyi	Luciferase structural genes	117-119
Xenorhabdus luminescens	Luciferase structural genes	120
Unidentified terrestrial bacterium	Luciferase structural genes	121
Beetle		
Luciola cruciata (Japanese firefly)	Luciferase cDNA	122
Luciola lateralis (firefly)	Luciferase cDNA	123
Luciola mingrelica (Eastern European firefly)	Luciferase cDNA	124
Photinus pyralis (North American firefly)	Luciferase cDNA	125
Pyrophorus plagiophthalamus (click beetle)	Luciferase cDNA	126
Other		
Aequorea species (jellyfish)	Aequorin cDNA	127
Vargula hilgendorfii (ostracod)	Luciferase cDNA	128
Renilla reniformis (sea pansy)	Luciferase cDNA	129
Clytia gregarium (hydroid)	Clytin cDNA	130
Obelia longissima (hydroid)	Obelin cDNA	131
Anthozoa species (nonbioluminescent)	cDNA	132

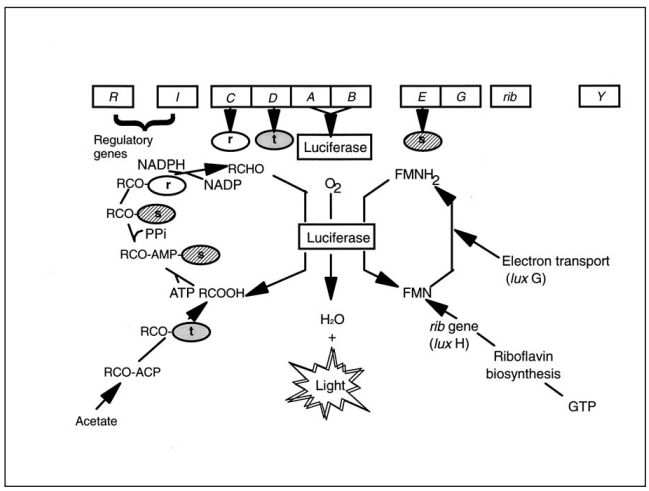

Figure 1) *Schematic representation of the genetics and biochemistry of bacterial bioluminescence. ACP Acyl carrier protein; AMP Adenosine monophosphate; ATP Adenosine triphosphate; H_20 Water; NADP Nicotinamide adenine dinucleotide phosphate; NADPH Reduced nicotinamide adenine dinucleotide phosphate; O_2 Oxygen; PPi Inorganic pyrophosphate; r Reductase; s Synthetase; t Transferase*

TABLE 2
Microorganisms engineered to obtain luminescent phenotypes

Species	Transfer system	Luminescent genes used	Reference
Gram-negative bacteria			
Aeromonas salmonicida	T	Vh *luxAB*	133
Agrobacterium radiobacter	C	Vh *luxAB*	134
Agrobacterium rhizogenes	C	Vh *luxAB*, Vf *luxABCDE*	134,135
Agrobacterium tumefaciens	C	Vh *luxAB*, Vf *luxABCDE*	134,135
Bradyrhizobium japonicum	C	Vh *luxAB*	22,136
Citrobacter koseri	Tf	Vf *luxAB*	46,137
Erwinia amylovora	C	Vf *luxABCDE*	135
Erwinia caratovora	C	Vf *luxABCDE*	135
Escherichia coli	C,Tf,Td	Vf Vh Pp Pl Xl *lux*, Lm Php *luc*	3,124,125
Escherichia coli O157	Tf,Td	Vf *luxAB*, Php *luc*, Pl *luxCDABE*	86,88,91
Klebsiella aerogenes	Tf	Vf *luxAB*	137
Pseudomonas aeruginosa	Tf	Vh *luxAB*	138
Pseudomonas fluorescens	C	Vf *luxABCDE*	135
Pseudomonas glumae	C	Vf *luxABCDE*	135
Pseudomonas putida	C	Vh *luxAB*	84,134
Pseudomonas syringae	C	Vf *luxABCDE*	135
Rhizobium meliloti	C	Vh *luxAB*, Vf *luxABCDE*	134,135
Rhizobium leguminosarium	C	Vh *luxAB*	134
Salmonella anatum	Tf,Td	Vf *luxAB*	61
Salmonella enteritidis	Tf,Td	Vf *luxAB*	61
Salmonella hadar	Tf	Pl *luxCDABE*	48
Salmonella typhimurium	Tf,Td	Vf *luxAB*	13,41,46
Shigella flexneri	Tf	Vf *luxAB*	46
Vibrio parahaemolyticus	Td	Vf *luxABCDE*	139
Yersinia enterocolitica	Tf	Vf *luxAB*, Lm *luc*	140
Gram-positive bacteria			
Bacillus megaterium	Tf	Vf *luxAB*	36
Bacillus subtilis	Tf	Vf *luxAB*, Vh *luxAB*, Php luc, Pyrp *luc*	36,141, 142
Bacillus thuringiensis	Tf	Vf *luxAB*	143
Campylobacter jejuni	Tf	Pl *luxAB*	144
Clostridium perfringens	Tf	Vf *luxAB*	145
Lactobacillus casei	Tf	Vf *luxAB*	146
Lactococcus lactis	Tf	Vf *luxAB*	146,147
Lactococcus lactis subspecies *diacetylactis*	Tf	Vf *luxAB*	146
Listeria monocytogenes	Tf	Vf *luxAB*	68,148
Mycobacterium tuberculosis	Tf,Td	Php *luc*	109
Mycobacterium smegmatis	Tf,Td	Php *luc*	110
Staphylococcus aureus	Tf,Td	Vf *luxAB*	29,105
Streptococcus thermophilus	Tf	Vh *luxAB*	149
Streptomyces coelicolor	Tf	Vh *luxAB*	20
Yeast			
Saccharomyces cerevisiae	Tf	Vh *luxAB*	150

C Conjugation; Lm Luciola mingrelica; Php Photinus pyralis*; Pl* Photobacterium leiognathi*; Pl* Photorhabdus (Xenorhabdus) luminescen*; Pp* Photobacterium phosphoreum*; Pyrp* Pyrophorus plagiophthalamus*; Tf Transformation; Td Transduction; Vf* Vibrio fischeri*; Vh* Vibrio harveyi

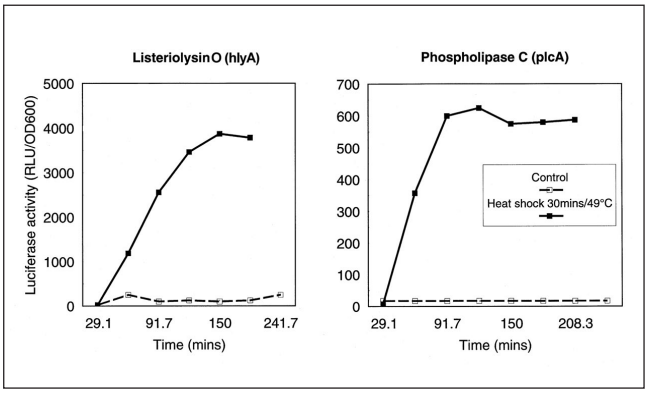

Figure 2) *Bioluminescence as a reporter of virulence factor gene expression in* Listeria monocytogenes*. Data from reference 34*

factor-associated genes can be made so that expression of the latter will be accompanied by light emission. Sheehan et al (29) used this approach to study the epidermolytic toxin (*eta*) gene of *Staphylococcus aureus,* which encodes a toxin causing the exfoliation of skin in neonates. They fused the promoter region of the *eta* gene with the *Photobacterium (Vibrio) fischeri luxAB* gene and found that expression of the *eta* gene was growth-phase dependent, being more rapidly transcribed during the late exponential to early stationary phase. The *eta* gene was also shown to be under the control of an accessory gene regulator. Furthermore, the effects of environmental conditions, such as osmotic strength, on the *eta* promoter could be assessed by this method. This technology provides a unique opportunity to study, in real time and nonintrusively, the control of epidermolytic toxin synthesis during the disease process using in vivo experimental models (16). A luciferase-based reporter system has also been used to study the expression of the toxic shock syndrome toxin-1 (*tst*) gene of *S aureus* (30), and the results suggested that a reduction in specific growth rate was the major factor controlling *tst* expression.

A haemolysin (listeriolysin O) and phospholipase C, encoded by genes designated *hlyA* and *plcA*, respectively, have been shown to play a role in the virulence of *Listeria monocytogenes* (31,32). These genes, along with others located in the virulence chromosomal region, are coordinately regulated by the product of the *prfA* gene (33). Park et al (34) constructed mutants of *L monocytogenes* in which the promoter sites of the *hlyA* and the *plcA* genes were fused with the *V fischeri luxAB* gene. They found that light emission was much higher in strains containing an intact copy of *prfA*, providing confirmation that the activity of these promoters was dependent upon the transcriptional activator, PrfA. They were also able to show that synthesis of listeriolysin O and phospholipase C was induced by heat shock (Figure 2), whereas oxidative stress had no effect on the expression of these virulence factors. In addition, the medium composition had a marked effect on the expression of the virulence genes. For example, it has been reported that the *hlyA* and *plcA* genes are repressed in the presence of cellobiose (35).

In the future, this technique will provide valuable information on how food composition and the environment in which

food is stored affect the pathogenicity of organisms responsible for foodborne illness, as well as shed light on events that occur when the pathogen enters the gastrointestinal tract.

Reporter of germination and sporulation: Luciferase has been used as a marker for gene expression in *Bacillus* species (36,37). Spores of *lux* recombinant bacteria do not luminesce until germination occurs, and this provides a valuable tool for the study of gene expression during both germination and sporulation. Another application for bioluminescent constructs of *Bacillus megaterium* involves the determination of the heat resistance of spores (38), which can be predicted because the level of light output from these mutants is directly proportional to the heat treatment received (39).

Reporter of cellular injury: Because the bacterial luciferase reaction requires a reduced form of flavin mononucleotide, any stimulus that affects the intracellular production of this substrate will result in a change in light output. This makes bioluminescence an effective tool for monitoring sublethal injury and subsequent recovery from the physiological insult (13,14,40). As an example, recovery from cellular injury caused by freezing has been studied using a *luxAB* recombinant of *Salmonella typhimurium* (41). Cells of the bacterium (10^5/mL in peptone water) were frozen at –20°C and, after thawing, they were compared with a culture of non-frozen cells by measuring bioluminescence and plate count. The data obtained by both methods were equivalent, but the bioluminescence measurements were obtained in real time. Interestingly, freezing brought about a 40-fold reduction in viable cells, whether measured by bioluminescence or by plate count, after a 2 h recovery period in Luria broth. However, the bioluminescence data indicated that, immediately after thawing, there was only a fivefold difference between control and frozen cultures (14). From these figures, Stewart (14) concluded that 20% of the *S typhimurium* population was able to survive the freeze-thaw cycle, with an intracellular biochemistry sufficiently intact to allow immediate light production, but only 2.5% of the cells were capable of division. In other words, a large proportion of cells could survive freezing with a functional metabolic system but these cells were not viable. Thus, bacteria that are sublethally injured in this manner could continue to produce toxigenic compounds, even though they remain nonculturable. The exact mechanism for induction of this so-called 'viable but nonculturable' (VNC) state is poorly understood, but vital information on the metabolic activity of VNC organisms can be provided by studying bioluminescent phenotypes (42,43). Duncan et al (44) followed the response of *Vibrio harveyi* and bioluminescent strains of *Escherichia coli* and *Pseudomonas fluorescens* to starvation, and found that quantification of luminescence changes enabled measurement of both culturable and VNC cells. Such information may allow the hypothesis of Bloomfield et al (45) to be tested; they suggest that the VNC phenomenon is the result of an imbalance in metabolism set up when stressed cells are suddenly transferred to a nutrient-rich medium. This leads to an almost instantaneous production of superoxide and free radicals which these nonadapted cells cannot detoxify, and, as a consequence, some or all of these cells die.

Ellison et al (41) also reported on the use of bioluminescence to elucidate the mechanism of freeze injury. They found that the rate of freezing affected the survival of *S typhimurium*. Survival rates in supercooled (–70°C) cultures were significantly lower than those in cultures frozen to –20°C at the same freezing rate. An osmoregulated *proU* promoter fused with *luxAB* (46) was used to confirm that freeze injury was the result of membrane damage, resulting in leakage and not accumulation of potassium ions (41).

Chen and Griffiths (47) used a luminescent strain of *Salmonella enteritidis*, with the *luxAB* genes chromosomally located, to monitor recovery from acid and heat shock. A culture of the luminescent salmonella was subjected to stress and allowed to recover in a nonselective medium at room temperature for 20 h. The cells could recover from exposure to either hydrochloric acid (pH 1.8) or acetic acid (pH 3.9) for 2 mins, and recover from heating at 55°C or 65°C for 60 mins. This approach was further adapted by Bautista et al (48). In their work, a luminescent strain of *Salmonella hadar* was constructed and used to inoculate turkey breast meat samples. These were then treated with lactic acid (4.25%, pH 3.0) at 40°C for 10 s, before storage at –12°C, 0°C, 5°C and 10°C. Viability of the salmonella was measured as light emitted from the bacterial cells directly on the turkey breast, and recovery from injury was estimated by monitoring light output after incubation at 22°C for 10 h. Unexpectedly, the lowest recovery rate was observed after storage at 5°C and the fastest recovery was on turkey breasts that had been stored frozen at –12°C. The ability to observe recovery of bacteria from insult directly on foods can provide much more information than studies performed in culture media.

During the early stdationary phase, bacterial cells acquire greater resistance to environmental stressors, such as heat, low pH and osmotic pressure, due to the induction of specific sets of genes by an RNA polymerase sigma factor (RpoS) encoded by the *rpoS* gene (49). There are several mechanisms at the transcriptional, post-transcriptional and post-translational levels for regulation of RpoS in *E coli* and *Salmonella* species (50). Thus, a reporter of *rpoS* transcription would not be a reliable indicator of intracellular levels of active RpoS. In some *Salmonella* serovars, it has been shown that a virulence determinant transcribed by the gene *spvA* is under the control of the product (SpvR) of the *spvR* gene (51). The *spvR* gene is, in turn, under the control of RpoS. With this in mind, Swift and Stewart (52) developed a *spv*::*lux* bioluminescence reporter of RpoS activity, and used this reporter to show that events under the control of RpoS could be induced in the presence of a competitive microflora at levels of 10^6 cells or more. Thus, in the presence of high levels of competing cells, salmonella acquired resistance to several environmental stresses (53). Stewart's group also used this reporter to demonstrate that they could monitor RpoS levels in cells in model food systems (54).

Bioluminescence has also provided a novel method for studying stress responses in foods at the molecular level. *Lux* gene fusions with the promoter sequences of genes involved in stress responses have been constructed in *E coli*, so that the organism emits light when it is under a stress that triggers transcription of the stress promoter:*luxCDABE* gene fusion (55-60)

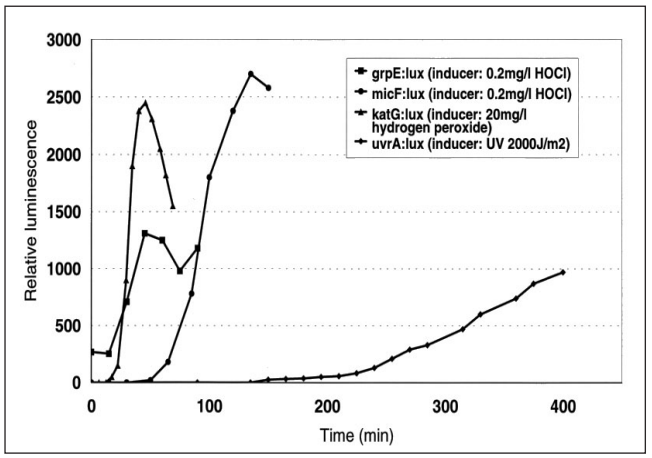

Figure 3) *Stress promoter gene::*lux *gene fusions to report on induction of stress in* Escherichia coli*. HOCl Hypochlorous acid. Data from references 55,57,60*

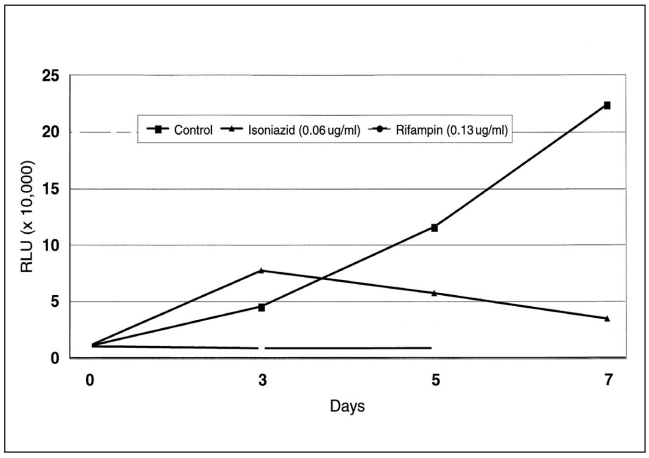

Figure 4) *Assay of susceptibility of intracellular* Mycobacterium tuberculosis *to isoniazid and rifampin using bioluminescence. Data from reference 76*

(Figure 3). Using these constructs, it has been shown that chlorine triggers an oxidative stress response, inducing activation of both heat shock and *soxRS* regulons within 1 s of exposure to free chlorine (57). Mutants of *E coli* carrying gene fusions of the *luxCDABE* genes with DNA damage-inducible promoters *recA*, *uvrA* and *alkA* may help in further elucidating the mechanisms involved in DNA repair (60). It is not difficult to envisage how powerful this technique will be when used in conjunction with imaging techniques that allow light emission to be detected directly in foods (48,61) and from single bacterial cells (24).

REPORTER OF BIOCIDE EFFICIENCY AND ANTIBIOTIC SUSCEPTIBILITY

Bioluminescence can be used to test rapidly the efficacy of sanitizers and disinfectants used in the food industry. Light emission from a *lux* recombinant organism is dependent on a functional intracellular metabolism, and any substance that interferes with these intracellular processes will result in a decrease in light production. These responses occur very rapidly, usually within 15 mins, giving a real time estimate of the efficiency of the sanitizing or disinfecting procedures (13,18, 19,62,63). It is possible to produce biosensors consisting of a genetically engineered bioluminescent reporter organism interfaced with an integrated circuit to report on the presence of microbial inhibitors (64). Indeed, bioluminescent bacteria have been used to assess the ability to clean food processing equipment effectively (65).

Walker et al (66) used bioluminescent constructs of *L monocytogenes* to measure the antilisterial activity of phenol and chlorhexidine diacetate. After determining the time for a $\log_{10}$ reduction in count or light output (D-value) at different biocide concentrations and plotting a double log plot of D-value against biocide concentration, the concentration exponents were calculated from the slope of the best fit line. Similar results were obtained when concentration exponents were assessed using plate count (8.3 for phenol and 2.13 for chlorhexidine) or the bioluminescent strains of *L monocytogenes* (7.3 for phenol and 2.63 for chlorhexidine). However, the results were obtained in 60 mins by the latter method. The *lux* recombinant *L monocytogenes* strains were also used to study the efficacy of the disinfectant Virkon (67), as well as other biocides including hypochlorite (68). Bacteria with bioluminescent phenotypes can also be used to measure antimicrobial activity against cells present as a biofilm, as well as the presence of environmental toxicants (14,18,69,70).

Agents that affect the integrity of cell membranes can also be assayed using bioluminescence. The click beetle luciferase gene has been cloned into several microorganisms, such as *E coli*, *Bacillus subtilis* and *Spodoptera frugiperda*. The luciferase enzyme encoded by the gene requires luciferin as a substrate. The diffusion of this substrate through cell membranes is slow at physiological pH and, therefore, a change in membrane permeability is seen as a change of in vivo luminescence of the cells (71). This technique has value for the assay of nisin and other membranolytic biocides and antibiotics.

Bioluminescence can be used to monitor the response of cell-wall deficient (L-forms) of *L monocytogenes* to biocides (72). These L-forms are difficult to culture, and the use of bioluminescence offers a near real time method for determining viability. L-form colonies required at least three days and as long as 21 days to become visible on agar plates, but they could be detected by bioluminescence after only 8 h of incubation.

The use of a *lux* recombinant bacterium for determining antibiotic susceptibilities was first described by Ulitzur and Kuhn (73). Since then, reporter strains of bacteria expressing firefly or bacterial luciferase have been used to evaluate the activities of antibiotics against *Mycobacterium tuberculosis* and *S typhimurium* in whole animal studies (74,75) and against mycobacteria sequestered in human macrophages (76) (Figure 4). This technique may also be useful when evaluating the actions of antibiotics against other intracellular pathogens such as *L monocytogenes*.

REPORTER OF ADHESION AND BIOFILM FORMATION

The adhesion of bacteria to surfaces has been studied by in vivo bioluminescence (69,77-80). Mittelman and co-workers (79,81) developed an on-line system for monitoring bacterial

adhesion by a *P fluorescens* strain containing the *V fischeri lux* gene operon. Both biofilm and bulk phase biomass could be assayed with a detection limit of 2 10^5 attached cells/cm^2. Light production was related to biofilm lipid synthesis per unit area. The method has potential for the study of bacterial adhesion to cells during infection and as an indicator of the bactericidal activity of host defence mechanisms (82).

The use of bioluminescence may help us understand the mechanisms of biofilm formation at the molecular level. A bioluminescent reporter plasmid, pUTK50, of alginic acid biosynthesis has been transconjugated into strains of *Pseudomonas putida*, *P fluorescens* and *Stenotrophomonas maltophilia* (83). When stimuli that increased alginate synthesis were applied to these strains, increased bioluminescence was observed, but exopolysaccharides other than alginate were produced. This suggests that the strains possessed homologous promoter sequences which control the genes necessary for the production of exopolysaccharides in response to environmental stimuli.

Bioluminescence also has application in the study of biofilms in the food industry. Dhir and Dodd (69) studied the resistance of substratum-attached, detached and planktonic cells of *S enteritidis* phage type 4 to biocides using in vivo bioluminescence. An excellent correlation between bioluminescence and classical plate count data was obtained when attachment profiles, biocide concentration exponents and thermal inactivation (D-values) were compared. There is a clear difference in the susceptibility to biocides of attached cells and those present in biofilms. Following treatment with a nonfoaming acid sanitizer or a liquid hypochlorite sanitizer, bioluminescent *P putida* cells present as a biofilm on n-buna rubber were able to recover after a 16 h resuscitation period and grow to levels approaching those of nontreated controls (84). Bioluminescence has also been used to demonstrate that L-forms of *L monocytogenes* are capable of forming biofilms on stainless steel and other surfaces (85).

Bioluminescence may also be applied in studies on the adhesion of bacteria to food. Siragusa et al (86) used a luminescent strain of *E coli* O157:H7 to study adhesion of the organism to beef carcass tissue. They found that retention of the bioluminescent signal was higher on lean fascia-covered tissue than on adipose fascia-covered tissue following a water rinse and that the luminescence was strongly correlated with bacterial counts.

REPORTER OF THE MICROBIAL ECOLOGY OF FOODS

The use of luminescence-based systems for studying microbial ecology has gained widespread acceptance by environmental microbiologists (87), and these techniques are now being applied to food. As well as providing quantitative information, imaging of luminescent bacterial cells can also provide information on their spatial distribution in a food. Chen et al (61) studied the ability of a *lux* recombinant of *S enteritidis* to penetrate the egg shell membrane and to grow in eggs under different storage conditions. This strain of *S enteritidis* was unable to penetrate the egg shell membrane but was able to grow in the space between the shell and membrane. The luminescent *Salmonella* strain could remain metabolically active in eggs after storage at 4°C for four weeks (61). The technique is nondestructive, gives real time results and can be carried out directly on the food of interest.

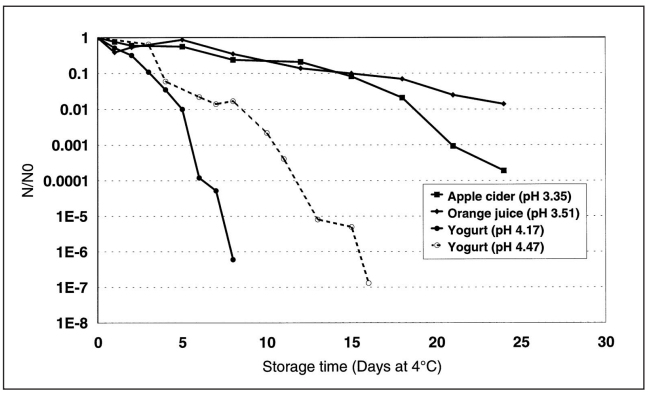

Figure 5) *Survival of luminescent phenotypes of* Escherichia coli *0157:H7 in apple juice (91), orange juice (91) and yogurt (88), determined by bioluminescence*

Luminescent and fluorescent strains of *L monocytogenes* and *E coli* O157:H7 have been used to monitor the survival of the organisms in yogurt and cheese (88,89), during a simulated fermented sausage manufacturing process (90), and in apple and orange juice (91) (Figure 5).

These survival studies indicate that bioluminescent reporter microorganisms have great potential as tools for developing quantitative risk assessment models. Already, thermal inactivation models for *S typhimurium* derived using bioluminescent strains have been shown to be equivalent to models calculated from plate count data (92,93) (Figure 6). This enables models to be generated in the presence of high levels of background microflora (92) and in real food systems (48).

REPORTER OF PATHOGENESIS

Several recent reports have illustrated the usefulness of bioluminescence to study pathogenicity (5). For example, Mettenleiter and Graewe (94) used a recombinant herpes virus carrying the firefly luciferase genes to monitor activity in single virus-infected cells. Verocytotoxicity of *E coli* cultures has been measured by transformation of Vero cells with a plasmid containing an intronless, firefly luciferase gene (95). Verotoxin could then be assayed by measuring the decrease in light emission with incubation time (Figure 7). The time taken for the assay could be reduced from three days for the conventional Vero cell cytotoxicity assay to 6 h for the bioluminescent cell technique, with no loss in sensitivity.

Arguably, the most exciting development is the ability to use bioluminescence to monitor bacterial pathogens in a living host. Contag et al (75,96,97) converted three strains of *S typhimurium*, which differed in their virulence for mice, to a bioluminescent phenotype through transformation with a plasmid conferring constitutive expression of bacterial luciferase. Using low light imaging, they were able to detect photons transmitted through tissues of the animals infected with the luminescent salmonella, and this allowed localization of the bacteria to specific tissues. Patterns of bioluminescence were observed

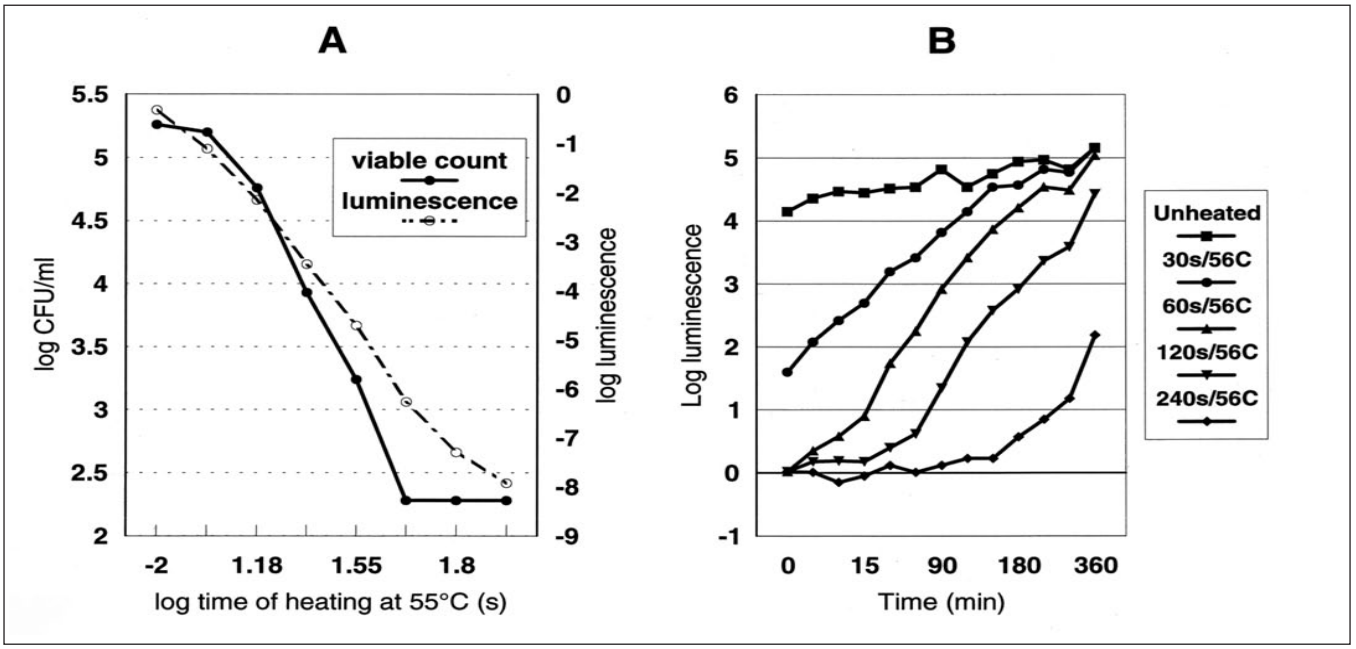

Figure 6) *Use of luminescent phenotype of* Salmonella typhimurium *to assess heat resistance.* **A** *Comparison of viable count and bioluminescence, data from reference 92; and* **B** *Monitoring recovery from heat treatment using bioluminescence, data from reference 93*

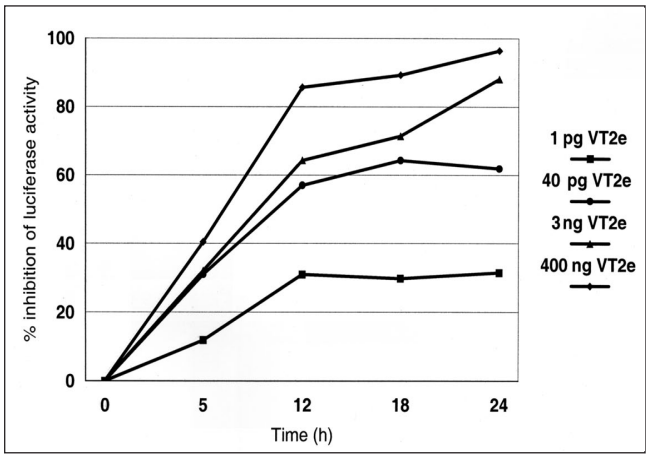

Figure 7) *A bioluminescence assay for verocytotoxin production by* Escherichia coli *0157:H7. Data from reference 95*

that suggested that the caecum played a vital role in salmonella pathogenesis. It was concluded that real time, noninvasive analyses of pathogenic events and pharmacological monitoring could be performed in vivo.

Bacteria are able to sense and respond to their own population densities by releasing and sensing pheromones. This phenomenon is referred to as 'quorum sensing' (98). In Gram-negative bacteria, quorum sensing is under the control of the LuxR family of transcriptional regulators, and the pheromone molecules (synthesized by members of the LuxI family of proteins) are acyl homoserine-lactone (AHSL) analogues. This form of cell-cell communication plays an important role in the regulation of expression of virulence factors involved in pathogenesis and luminescence and may be an important tool for studying these regulatory mechanisms (99). Because pathogenic bacteria use AHSL signals to regulate virulence genes, an understanding of the underlying mechanisms of quorum sensing may lead to the development of novel antivirulence drugs (100). The link between virulence and cell density was demonstrated using a bioluminescent reporter, where it was shown that *S enteritidis* strains that grew to a higher cell density than the wild type underwent significant morphological changes and became more virulent (101).

BACTERIAL DETECTION USING MOLECULAR BIOLUMINESCENCE

Ulitzur and Kuhn (73) proposed an elegant method for the detection of bacteria in foods based on the innate specificity of bacteriophage for an appropriate bacterial host. They cloned the *lux* genes into host-specific phages so that, on infection, the bioluminescent genes would be transferred to the host bacterium where they would be expressed, causing light to be produced. Light is not produced until after infection by the phage because the phage does not possess the intracellular biochemistry to express the genes and the host does not contain the *lux* genes until transduction occurs. This detection method can be quantitative if the phage is present at optimal concentrations because the amount of light emitted is proportional to the number of infected bacteria. This methodology has been applied to the detection of a variety of bacteria and can even be used for the detection of bacteria directly in foods (102).

Detection of *E coli*: In their original work, Ulitzur and Kuhn (73) used cell concentrations of *E coli* W3110 in the range 10^1 to 10^4 colony forming units (cfu)/mL. The *E coli* cells were concentrated by membrane filtration and the membranes were placed in scintillation vials. Following addition of a *lux* modified L4 phage (7 10^7 plaque forming units [pfu]), luminescence in the vials was measured after incubation for 40, 60 and 100 mins at 20°C. The level of light produced was directly related to the initial number of *E coli* cells present in the sam-

ple, and as few as 10 cells in the sample could be detected within 100 mins. The *lux* L4 phage (2 10^8 pfu) was also added to *E coli* W3110 cells in sterile milk and luminescence was measured after incubation at 25°C for 18, 27 and 40 mins (73). Again, initial numbers of *E coli* cells in milk were directly correlated with light production, and the assay could detect 10 *E coli* cells/mL of milk within 30 mins. The method was also capable of detecting and enumerating *E coli* cells in urine samples.

It has been estimated that there are over 100,000 cases of illness caused by enterohemorrhagic *E coli* annually in the United States, resulting in nearly 80 deaths (103). This has made it desirable to develop simple and rapid methods for the detection of this organism, and bacteriophage-based methods may prove to be valuable. A bacteriophage broadly specific for *E coli* O157, designated AR1, has been isolated (104). A *Luciola mingrelica luc* recombinant AR1 has been obtained by simultaneously infecting wild type *E coli* O157:H7 with a pBluescript II SK (±) phagemid containing the *luc* gene (Promega Corporation, Madison, Wisconsin) and AR1 (105). The transducing phage was capable of detecting 10^6 *E coli* O157:H7 cells/mL.

Detection of *Salmonella* species: A *lux*$^+$ P22 phage has been engineered and used in experiments to infect *S typhimurium* LT2 (19). When light emission following infection was measured with a luminometer, as few as 10^2 *S typhimurium* cells could be detected, even when they were present in mixed culture at a ratio of one salmonella cell to 10^6 cells of other bacteria. *S typhimurium* could be detected with the *lux*-modified P22 phage in environmental samples including water, soil and sewage sludge (106). These researchers adopted a most probable number technique based on a 15 tube test method, consisting of five tubes containing 10 mL, 1 mL or 0.1 mL of sample in buffered peptone water. After overnight incubation at 37°C, subsamples from each tube were transferred to Luria broth in scintillation vials and 6.9 x 10^9 pfu of the *lux*-modified P22 phage added. Light output was measured with a luminometer after 90 mins incubation at 30°C. There was an excellent correlation between the most probable number obtained by the luminescence method and plate count for all samples, and results were achieved within 24 h. No false positive or false negative results were obtained with the *lux*$^+$ phage method in any of the samples tested.

Chen and Griffiths (102) obtained three recombinant phages carrying the *luxAB* genes from *V harveyi*, either by infection of luminescent strains of *Salmonella* with wild type bacteriophage or by bacteriophage induction from luminescent, lysogenic bacterial cells. Using these phages, it was possible to detect, by photon imaging techniques, light emitted after infection of group B, D and some group C *Salmonella* species directly in milk, on the surface of chicken meat and even in whole eggs. Eggs contaminated with about 10^3 cfu/egg became luminescent after 16 h incubation, but the detection limit could be decreased to as few as 63 salmonella cells/egg when the incubation period was extended to 24 h. Chen and Griffiths (102) also described a technique whereby Petrifilm (3M, St Paul, Minnesota) and the *lux*$^+$modified phage could be combined for the detection of *Salmonella* species using x-ray autoradiography for observing light emission.

Detection of *L monocytogenes*: Loessner et al (107) isolated a bacteriophage, designated A511, which is a *Listeria* genus-specific, virulent myovirus that infects 95% of *L monocytogenes* one-half and four serovars. A recombinant phage was constructed by homologous recombination, which carried the gene for a fused *V harveyi* LuxAB protein inserted immediately downstream of the major capsid protein gene (cps). Transcription of the *luxAB* gene was initiated by the cps promoter at 15 to 20 mins after infection, and infected cells were detectable by their bioluminescent phenotype. Following infection and a 2 h incubation period, between 10^2 and 10^3 *L monocytogenes* cells/mL could be detected using a simple luminometer. Loessner and his colleagues (108) subsequently evaluated the use of the A511::*luxAB* phage for testing contaminated foods and environmental samples for the presence of viable listeria cells. With a short pre-enrichment step of 20 h, the phage was capable of detecting very low initial contamination rates in several foods artificially contaminated with *L monocytogenes* Scott A cells. In ricotta cheese, chocolate pudding and cabbage, less than one cell/g of food could be detected by comparing the light emission of phage-infected samples to that of controls without the *lux*$^+$ phage. In foods having a large and complex microbial background flora, such as ground beef and soft cheese, at least 10 cells/g were necessary to produce a positive bioluminescence signal. Of 348 potentially contaminated natural food and environmental samples, 55 were found to be listeria positive by the *lux* phage assay versus the 57 positive samples detected by the standard plating procedure. The *lux* phage procedure detected more positive samples among dairy products and environmental samples, whereas the plating procedure revealed more contaminated meat and poultry samples. Overall, both methods were equally sensitive. However, the minimum time required for detection of listeria with the *lux* phage assay was 24 h, as opposed to the four days needed by the standard plating method. The phage could also be used in a most probable number technique to provide rapid enumeration of low levels of listeria cells in foods against the background of a competing microflora.

Detection of *M tuberculosis*: Although foodborne tuberculosis infection has been eradicated in Western countries, the numbers of reported tuberculosis cases are again on the rise, mainly due to the emergence of antibiotic-resistant strains of the organism. Culture methods for *M tuberculosis* require several weeks, and the need for more rapid testing systems prompted Jacobs et al (109) to investigate bioluminescence. Shuttle phasmid vectors from a number of mycobacteriophages can efficiently deliver recombinant DNA into mycobacteria by infection, and these phasmids are also amenable to genetic manipulation in *E coli*. A shuttle phasmid from a mycobacteriophage (TM4) that formed plaques, not only on the slow growing *M tuberculosis* but also on the faster growing species *Mycobacterium smegmatis,* was constructed by inserting the firefly *luc* gene downstream of a strong *hsp*60 promoter in an *E coli* cosmid (109). The *luc*$^+$ phage infected Bacillus Calmette-Guérin (BCG) vaccine strains of mycobacteria, as well as *M tuberculosis* and *M smegmatis.* When mixed in approximately equal numbers (5 10^7 cfu/mL bacteria and 5 10^7

TABLE 3
Properties of luciferases from different sources

Luciferase	Substrate	Light emission wavelength (nm)
Bacterial	Long chain aldehyde	490
Firefly	Luciferin	560
Click beetle	Luciferin	548 to 593

pfu of phage), light was produced within minutes of infection, although the limit of detection appeared to be 10^4 cfu/mL. The light output was increased 1000-fold following incubation for 2 h at 37°C but was still much lower than the light emitted by an equal number of cells transformed with the *luc*$^+$ plasmid. This was probably due to less efficient gene expression in phage-infected cells or phage-mediated inhibition of the cellular metabolism in the host bacterium, resulting in lower intracellular ATP concentrations. The slower growth of *M tuberculosis* compared with *M smegmatis* was probably not caused by differences in gene expression because the kinetics of light production were the same in both species (109). It has been claimed that as few as 10 cells of *M smegmatis* can be detected in 40 h using L5 luciferase reporter mycobacteriophage (110).

The versatility of molecular bioluminescence was demonstrated by Jacobs et al (109), who used the bioluminescent *M tuberculosis* cells obtained by transduction to determine their antibiotic susceptibility. An antibiotic-sensitive strain, an isoniazid-resistant strain and a multiple drug-resistant strain of *M tuberculosis* were grown and, following incubation with antibiotics, the transducing particles were added to give a multiplicity of infection of 1000. Luciferin was added after a further incubation step and light production was measured in a luminometer. Resistance to an antibiotic was demonstrated by light production, whereas strains sensitive to the test drug did not luminesce. This methodology allowed a dramatic reduction in the time needed to determine the antibiotic susceptibility of *M tuberculosis* from two to three months by cultural methods to two to three days by bioluminescence.

Thus, one assay permits both rapid detection and antibiotic susceptibility of the actual causative agent of the disease. This is of considerable importance given the increase in antibiotic-resistant organisms and the emergence of a number of life-threatening foodborne pathogens, such as *S typhimurium* DT104, which possess increased drug resistance.

Detection of *S aureus*: In an independent study, the author's research group at Guelph has adopted the same strategy as Jacobs et al (109) to detect and determine the antibiotic susceptibilities of mastitis-causing agents, especially *S aureus* (105). A battery of five phages were identified that could infect all strains of *S aureus* tested, whether they were isolated from mastitic cows or from foods implicated in foodborne illness. A plasmid incorporating the *luxAB* genes fused to a staphylococcal cadmium-resistant gene promoter was introduced into staphylococcal phages by homologous recombination. Using these recombinant phages, it was possible to detect 10^6 *S aureus* cells/mL in broth cultures. The luminescent strains obtained after transduction could be used to test for antibiotic susceptibility.

Multiplex assays: The luciferases encoded by different organisms have distinct properties (1,111,112) which can be used to develop systems that can detect, and differentiate between, more than one bacterium in a single assay. For example, the luciferase encoded by the bacterial *lux* genes has a requirement for a long chain aldehyde and emits light at a wavelength of about 490 nm, whereas the firefly *luc* gene encodes an enzyme which uses luciferin and produces light at a wavelength near 560 nm (Table 3). Thus, the light produced from recombinant bacteria containing either the *lux* or *luc* genes can be easily distinguished, either on the basis of substrate specificity or emission wavelength. *Lux*-modified phages specific for *Salmonella* species and *S aureus*, and *luc*$^+$ phages that infect only *E coli* O157 strains have been engineered (102,105). With the aid of a 500 nm cut-off filter, it is easily possible to differentiate between bacteria expressing the *lux* genes and those expressing *luc* by photon imaging.

This method of differentiating between microorganisms has great potential for studying microbial interactions directly in food systems, and work in this area is being conducted in the author's laboratory.

Advantages and limitations of the method: *Lux*-modified phage-based detection methods are attractive to the food microbiologist because they offer a nondestructive, rapid and easy-to-use alternative to other technologies. A particularly attractive feature of these techniques is their ability to detect the presence of luminescent microorganisms directly on food surfaces through instrumentation that allows low light imaging. Although Ulitzur and Kuhn (73) have claimed that it is possible to detect as few as 10 bacterial cells/mL directly in milk using transduction of *lux* genes, no research has been published that confirms this observation. However, even if the method is unable to detect directly the low number of pathogens that may be present in food, the system is sensitive enough that dramatic reductions in enrichment times can be achieved (106,113). As discussed previously, recent work in the author's laboratory has shown that simple techniques can be used to differentiate between organisms expressing luciferase genes from different sources (eg, *lux* and *luc* genes). The application of this method to food opens the door for the development of 'single shot' assays capable of detecting more than one type of bacterium. The economy of this approach, in terms of both reagents and labour, would be appealing to food microbiologists.

Some of the difficulties that have to be overcome are associated with propagation and stability of the phage, as well as poor expression of the luciferase genes in the host. This is especially true for Gram-positive bacteria, but the use of *luxAB* gene fusions may go some way to alleviating this problem. The identification of suitable gene promoters to regulate expression of the luciferase gene is also a key factor in optimizing the phage-mediated assay.

The specificity of the method is only limited by the identification of suitable bacteriophages capable of only infecting the target bacterium. Bacteriophages generally have a narrow host

range, which is limited in most cases to single bacterial species. For certain bacteria, such as *Salmonella* species, phages are well characterized, but in other species, phages have still to be identified that infect an acceptable proportion of strains. Even when suitable bacteriophages are available, the lack of information on the phage genome can be a hindrance to the development of suitable assays.

CONCLUSIONS

The phenomemon of bioluminescence can be used in several ways to provide data that can help the food microbiologist achieve the goal of safer food. It can provide a way of detecting potential pathogens in food through the use of *lux*-modified phage. By creating organisms with a bioluminescent phenotype, it is possible to gain information on the way foodborne pathogens survive in food and interact with their environment. The potential of the technique has not been fully realized, but interest in applying molecular bioluminescence to solve problems related to the microbiological safety of foods is increasing.

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