Bioluminescence lights the way to food safety

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ABSTRACT

The food industry is increasingly adopting food safety and quality management systems that are more proactive and preventive than those used in the past, which have tended to rely on end product testing and visual inspection. The regulatory agencies in many countries are promoting one such management tool, Hazard Analysis Critical Control Point (HACCP), as a way to achieve a safer food supply and as a basis for harmonization of trading standards. Verification that the process is safe must involve microbiological testing but the results are not, as yet, generated in real-time. Of all the rapid microbiological tests currently available, the only ones that come close to offering real-time results are bioluminescence-based methods.

Recent developments in the application of bioluminescence for food safety issues are presented in this paper. These include the use of genetically engineered microorganisms with bioluminescent and fluorescent phenotypes as a real time indicator of physiological status of bacterial cells and survival of food-borne pathogens in food and food processing environments as well as novel bioluminescent-based methods for rapid detection of pathogens in food and environmental samples. Advantages and pitfalls of the methods are discussed.

Keywords: Food safety, microbiological testing, bioluminescent-based methods, real-time pathogen detection

1. INTRODUCTION

In vivo bioluminescence and *in vivo* fluorescence involve emission of visible light by living organisms through a series of enzyme-catalyzed reactions or upon excitation with UV or short wavelength light, respectively. Luminescent organisms are found in several ecological niches and comprise species of bacteria, dinoflagellates, fungi, protozoa, sponges, jellyfish, corals, squid, starfish, worms, fish and beetles (1). The biochemistry and genetics of light emission has been widely studied and now is being explored by scientists in other disciplines.

The genes responsible for *in vivo* bioluminescence and *in vivo* fluorescence have been identified and cloned. These genes have been transferred and expressed in different microorganisms, including several food-borne pathogens. Luminescent phenotypes have been obtained for both Gram-positive and Gram-negative bacteria and yeasts (Table 1). This review will describe data, obtained both by the authors at the Canadian Research Institute for Food Safety and available in the literature, on the application of bioluminescent bacteria to resolve food safety issues.

Table 1. Microorganisms engineered to obtain luminescent phenotypes

Species	Luminescent gene used	Reference
Gram-negative bacteria		
Aeromonas salmonicida	Vh *lux*AB	2
Agrobacterium radiobacter	Vh *lux*AB	3
A. rhizogenes	Vh *lux*AB, Vf *lux*ABCDE	3,4
A. tumefaciens	Vh *lux*AB, Vf *lux*ABCDE	3,4
Bradyrhizobium japonicum	Vh *lux*AB	5,6
Campylobacter jejuni	Phl *lux* AB	28, 29
Citrobacter koseri	Vf *lux*AB	7,8
Erwinia amylovora	Vf *lux*ABCDE	4
E. caratovora	Vf *lux*ABCDE	4
Escherichia coli	Vf Vh Pp Pl Phl *lux* AB, *lux* CDABE; Lm Php *luc*	9-11, 51
E.coli O157:H7	Vf *lux* AB, Php *luc* Phl *lux* CDABE	12-14
Klebsiella aerogenes	Vf *lux* AB	8

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Pseudomonas aeroginosa	Vh *lux* AB, *lux* CDABE	15, 47
Ps. fluorescens	Vf *lux* ABCDE. Phl *luxCDABE*, Vf *lux* ABCDE	4,44,49, 56
Ps.glumae	Vf *lux* ABCDE	4
Ps. putida	Vh *lux* AB, Vf *lux* ABCDE	3,16,49,52
Ps. syringae	Vf *lux* ABCDE	4
Ralstonia eutropha	*lux* CDABE	53
Rhizobium meliloti	Vf *lux* ABCDE, Vh *lux* AB	3,4
Rh. leguminosarium	Vh *lux* AB	3
Salmonella Anatum	Vf *lux* AB	17
Salm. Enteritidis	Vf *lux* AB, Phl *lux* CDABE	17, 50
Salm. Hadar	Phl *luxCDABE*	18
Salm. Typhimurium	Vf *lux* AB, Phlg *lux*CDABE; *gfp*-UV	7, 19,20, 46
Shigella flexneri	Vf *lux* AB	7
Vibrio parahaemolyticus	Vf *lux* ABCDE	21
Yersinia enterocolitica	Vf *lux* AB, Phl *lux* CDABE, Lm *luc*	22,23
Gram-positive bacteria		
Bacillus megaterium	Vf *lux* AB	24-26
B. subtilis	Vf *lux* AB, Vh *lux* AB. Php *luc*, Pyrp *luc*	
B. thuringiensis	Vf *lux* AB	27
Clostridium perfringens	Vf *lux* AB	30, 55
Lactobacillus casei	Vf *lux* AB	31
Lactococcus lactis	Vf *lux* AB	31,32
L. lactis subsp. *Diacetylactis*	Vf *lux* AB	31
Listeria monocytogenes	Vf *lux* AB	33,34, 63
Mycobacterium bovis	Php *luc*	45
Mycobacterium tuberculosis	Php *luc*	35
M. smegmatis	Php *luc*	36
Staphylococcus aureus	Vf *lux* AB; Php *luc*	37,38; 43
Streptococcus thermophilus	Vh *lux* AB	39
Str. pneumonia	Phl *lux* CDABE	40, 48
Streptomyces coelicolor	Vh *lux* AB	41
Yeast		
Saccharomyces cerevisiae	Vh *lux* AB; Ppy *luc*	42, 54

2. LUMINESCENT BACTERIA AS CELL REPORTERS

2.1. Reporter of the microbial ecology of foods. The use of luminescence based systems for studying microbial ecology has gained widespread acceptance by environmental microbiologists 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. This technique is non-destructive, gives real-time results and can be carried out directly on the food of interest. In our previous work (17) it was shown that a *lux* recombinant strain of *Salmonella* Enteritidis was unable to penetrate the egg shell membrane but was able to grow in the space between the eggshell and the membrane. The luminescent *Salmonella* strain could remain metabolically active in eggs after storage at 4°C for 4 weeks (Figure 1). Similar research was carried out in our laboratory recently with a luminescent strain of *Campylobacter jejuni* (29). It was shown that *C .jejuni* was able to penetrate normal eggshells. Fresh eggs were more heavily colonized than retail eggs.

Luminescent and fluorescent strains of *Listeria monocytogenes* and *E. coli* O157:H7 have been used to monitor survival of the organisms in yogurt and cheese (13, 57), during a simulated fermented sausage manufacturing process (58) and in

apple and orange juice (14). Extensive growth of a bioluminescent strain of *Yersinia enterocolitica* on Camembert cheese at 10°C was monitored *in situ* by bioluminescent imaging with high sensitivity (23).

Luminescent bacterial cultures were used for studying attachment of bacteria to surfaces and to validate decontamination procedures in the food industry. Adherence of *Escherichia coli* O157:H7 to beef carcass surface tissues was monitored by using a constructed bioluminescent reporter strain (12). It was shown that retention of the pathogen after water rinses was significantly higher on lean tissue than on adipose tissue. In other work (59), it was demonstrated that irreversible attachment of *E. coli* to loin cuts was independent of whether the carcass had undergone a steam pasterization treatment. Use of bioluminescent *E. coli* showed that cells bound preferentially to cut edges and convoluted areas on the loin surface, and could not be removed by rinsing. A similar trend was shown for the survival of *E. coli* O157:H7 during the manufacture of fermented sausage (Figure 2). The bioluminescent signal originating from the recombinant strain of *E. coli* was associated mostly with adipose tissue, while lactic acid bacteria were located predominantly in lean and connective tissues (88).

Translocation of pathogenic bacteria from soil or water to plants is of great concern to fresh vegetable producers. With the help of pathogen bacteria transformed with plasmids encoding for green fluorescent protein and laser scanning confocal microscopy it was shown that contamination of seeds and plants resulted in presence of target bacteria not only on the surface of fresh produce but up to 40-70 μ m in depth (89-91). These results suggest that pathogens located in the internal structures or within plant/fruit tissues may evade decontamination treatment and pose a hazard for consumers. Due to the very high stability of green fluorescent protein in harsh environmental conditions it was impossible to distinguish between live and dead bacteria using this approach. In our laboratory, a bioluminescent strain of *Salmonella* Enteritidis was used to contaminate irrigation water applied to tomato plants. Preliminary experiments showed that substantial numbers of the bioluminescent pathogen survived in the soil, however, no live bioluminescent bacteria were found in leaves, stems and fruits of the plants.

2.2. Reporters of biocide efficiency and antibiotic susceptibility. Bioluminescent bacteria can be used to rapidly test the efficacy of sanitizers and disinfectants used in the food industry, as well as for validation of the cleaning procedure. 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 output. These responses occur very rapidly, usually within 15 minutes, giving a real-time estimate of the efficiency of sanitizing or disinfection procedures (19,60). Use of bioluminescent cultures proved especially effective when comparing the efficacy of biocides for planktonic and bacterial cells in biofilms (61,62). For *Salmonella* Enteritidis it was shown that cleansing regimes developed using planktonic cell data were effective against surface-attached cells of this bacterium (61). Attachment and viability of bacterial cells on different surfaces were investigated with the help of bioluminescent strains of *Pseudomonas putida* (16), *Pseudomonas aeroginosa* (47), *Listeria monocytogenes* (63). Bioluminescent reporter strains of *E. coli* carrying *lux* CDABE operon and *luc* genes (64-66) were used to detect traces of antimicrobial agents in fish and pork in real time. A highly bioluminescent *Streptococcus thermophilus* strain was constructed for the detection of dairy relevant antibiotics in milk (39). These methods were found to be reliable, sensitive, and allow results to be obtained in close to real-time format (2 h).

2.3. Reporters of cell injury and recovery. Since bioluminescent reactions require such metabolites as $FMNH_2$ or ATP, *in vivo* bioluminescent signals strongly depend on the metabolic status of cells. This makes bioluminescence an effective tool for monitoring sub-lethal injury and subsequent recovery from physiological stresses. Of special interest to the food microbiologist is the ability to monitor these processes directly in food, which makes the results much more relevant. As an example, recovery from cellular injury caused by freezing has been studied using *luxAB* recombinant *Salmonella* Typhimurium (20). Cells of this 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 by plate count. It was concluded that only 2.5% of the cells survived freezing were capable of division and growth. However, 20% of the cells where in a viable but non-culturable (VNC) state, characterized by cells with a functional metabolic system that continued to produce bioluminescence and toxigenic compounds but were unable to grow. The exact mechanism of this VNC state is poorly understood, but information vital for monitoring this state could be obtained by using bacteria with luminescent phenotype. An understanding of factors leading to cells entering the VNC state is very important for assessing cleaning regimes in food processing plants and for the development of strategies to eliminate pathogens from food products. In our laboratory, a luminescent strain of *Salmonella* Enteritidis with *lux*AB genes chromosomally-located has been used to monitor recovery from acid and heat shock (67). The cells could recover from exposure to either HCl, pH 1.8 or acetic acid, pH 3.9 for 2 minutes and also recover from heating at 55°C or 65°C for 60 minutes. This approach was further adapted to real food samples (18). A luminescent strain of *Salmonella* Hadar was constructed

and used to inoculate turkey breast meat. These samples were treated with lactic acid (pH 3.0) at 40°C for 10 sec before storage at –12, 0, 5, and 10°C. The viability of *Salmonella* was measured as light emitted from the bacterial cells directly on the turkey breast. Recovery from injury was monitored as light output during incubation at 22°C for 10 hours. Unexpectedly, the lowest recovery rate was observed after storage at 5°C, and the fastest after storage at -12°C. This is a good example of how bioluminescence can provide vital information that could not be obtained with studies performed in culture media.

2.4. Reporters of gene expression. In foods and food processing environments, microorganisms are exposed to various stress conditions such as osmotic shock, heat and cold shock, extreme pH and water activity. In many cases, expression of virulence genes can be initiated by stress conditions. Information of how food composition, and the conditions under which food is stored, affects the pathogenicity of organisms responsible for food borne illness is of high importance for food safety specialists. Bioluminescent reporter genes provide a unique opportunity to monitor target gene expression in real-time *in situ* (68). This approach was used to study the expression of epidermolytic toxin (*eta*) gene of *Staphylococcus aureus* (37). The promoter region of the *eta* gene was fused with the *lux*AB gene from *Photobacterium fisheri* and its expression was monitored in real-time. There was a higher expression of the toxin gene during the late-exponential and early stationary phase of growth. Furthermore, the effect of environmental conditions, such as osmotic strength, on the expression of the *eta* gene was assessed using this non-destructive, real-time method. For *Listeria monocytogenes*, it was shown that synthesis of the virulence factors listeriolysin O and phospholipase C was induced by heat-shock, whereas oxidative stress had no effect on the expression of these virulence genes (69).

2.5. Reporters of virulence and pathogenicity. Several recent reports have illustrated the usefulness of bioluminescent cells to study pathogenicity. For example, verocytotoxicity of *E. coli* cultures could be measured using Vero cells transformed with a plasmid containing an intronless firefly luciferase gene (70). The presence of verotoxin could be assayed by measuring the decrease in light emission over time. The most exciting development is the ability to use bioluminescence to monitor bacterial pathogens directly in a living host. Contag et al (68,71) infected mice with three *Salmonella* Typhimurium strains which differed in their virulence. Using a low-light imaging system, they were able to detect photons transmitted through tissues of the animals and monitor temporal and spatial localization of the luminescent bacterial pathogen. In was concluded that real time, non-invasive analysis of pathogenic events could be performed *in vivo*. This approach was used in our laboratory to investigate the role of diet on time course of infection in mice (72). Both the intensity of bioluminescence from mice and their physical condition indicated that animals fed with fermented milk, or fermented milk components, prior to infection were less susceptible to bacterial colonization and subsequent bacteremia (Figure 3). The use of bioluminescent pathogens is a promising model for assessing the effect of foods on the course of infection in live animals. It allows results to be obtained much faster and eliminates the need to sacrifice a lot of animals.

It has been established that bacteria are able to sense and respond to their own population density by releasing and responding to bacterial pheromones. This phenomenon was first described for the bioluminescent marine bacterium, *Vibrio fisheri*, and is referred to as quorum sensing (73). 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 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. Bioluminescence is an important tool for studying these regulatory mechanisms (74). The link between virulence and cell-density has been demonstrated using a bioluminescent reporter, when it was shown that *Salmonella* Enteritidis strains that grow to a higher cell density than wild type underwent significant morphological changes and become more virulent (75). Intra-species and interspecies cell-cell communication that regulates gene expression in bacterial pathogens could have very serious implications for food safety, which is why research on quorum sensing is of high priority for food microbiologists.

3. DETECTION OF PATHOGENIC BACTERIA USING BIOLUMINESCENCE

3.1. Bacteriophage-based techniques for bacteria detection. Enumeration of bacteria using *in vivo* bioluminescence is the most sensitive and accurate among the so-called rapid methods. However, only a limited number of bacteria in nature possess a bioluminescent phenotype. The three ways to introduce foreign DNA into bacterial cells are transformation, conjugation and transduction. The entry of DNA via transformation or transfection is expected to lack specificity since many organisms can accept extracellular DNA. Conjugation as a method for introducing DNA suffers from similar problems. These difficulties can be partly overcome by using bacteriophages as vectors of transmission. Specific transfection of bacteria with bioluminescent genes using recombinant bacteriophages was

proposed by Ulitzur and Kuhn as a method for detection of bacteria in foods (76). The phages are not able to express the genes, so they remain dark. However, when the phage infects the host cell, the luciferase is synthesized and the *E. coli* cells light up and can be detected with a luminometer. This approach was used to construct recombinant bacteriophages for the detection of *Enterobactereaceae* (77), *Listeria monocytogenes* (78,79), *Salmonella* (80-82), and *Mycobacterium tuberculosis* (83). A phage-based bioluminescent test for *Listeria* identified viable pathogen cells in ricotta cheese, chocolate pudding and cabbage at a level of less then one cell per g of food after a 20 h pre-incubation. The system allowed *Salmonella* cells to be detected directly in artificially contaminated whole eggs. In our laboratory, it was shown that after incubation for 24 h, eggs inoculated with 10^2-10^3 CFU per egg became luminescent (82). An additional advantage of recombinant phage technology for pathogen detection is that it can be performed under field conditions using a simple photographic film-based device for light detection (83).

In the earlier publications mentioned above, insertion of *lux* genes into the phage genome was performed by transposition using a transposon with a selectable marker, such as antibiotic resistance. This method is rapid but rather hit or miss. If the phage lacks non-essential genes or the packaging limits of the phage head are very strict, then the addition of several kilobases may lead to the loss of viability. In this situation, it is preferable to gain some knowledge of the molecular structure of the phage to allow non-essential sites to be identified for cloning the reporter genes into the phage DNA. Kuhn et al. (84) cloned and sequenced part of the genome of Felix 01 phage that is considered the best choice with regard to *Salmonella* due to its ability to lyse almost all *Salmonella* strains. This information was used by the same authors (85) while developing recombinant phage reagent for *Salmonella*. A non-essential gene discovered earlier allowed substitution of foreign DNA in a way that neither increased the size of the phage genome nor allowed loss of the insert by recombination.

3.2. Multiplex assays. The luciferases encoded by different organisms have different properties and emit light of different colour. This can be utilized to develop systems that can detect and differentiate between more than one bacterium in a single assay. For example, the luciferase encoded by bacterial *lux* genes has a requirement for a long chain aldehyde and emits blue light at a wavelength of about 490 nm. The firefly *luc* genes encode an enzyme which utilizes luciferin and produces light in the range from green to red depending on the species and structure (86). Using the proper cut-off filter, or colour sensitive light detection system, could allow simultaneous detection of several types of bacteria emitting light of different colour. A *Staph. aureus*-specific lux-modified phage was engineered in our laboratory, together with an *E. coli* O157:H7 specific phage carrying *luc* genes (87). With the aid of a 500 nm cut-off filter, it was easy to differentiate between bacteria expressing *luc* or *lux* genes (Figure 4). This method has great potential for studying microbial interaction directly in food systems and work in this area is being conducted in our Institute.

Figure 1 Bioluminescent image of the egg inoculated with 10^3 CFU of *Salmonella* sp and incubated at 37°C for 24 hours (17). Bioluminescence was monitored using BIQ Bioview Image Quantifier.

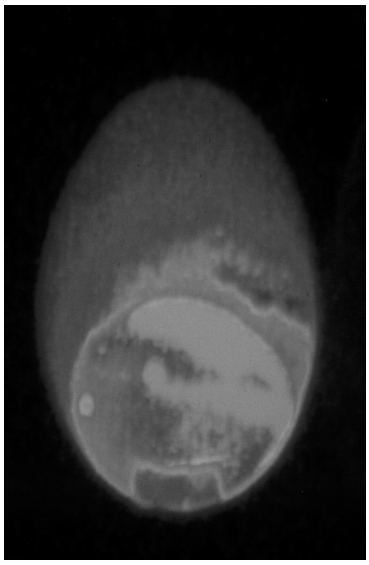


Figure 2. Survival of bioluminescent *E.coli* O157:H7 in fermented sausage: overlay of bioluminescent image of *E.coli* O157:H7 colonies on photo image of the slice of fermented sausage

Figure 3. Typical bioluminescent images of the mice infected with 10^6 cells of *Salmonella* Enteritidis on the 8th day after infection. 1 - control group; 2 - group fed with cell-free fraction of fermented milk; 3 - group fed with whole fermented milk.

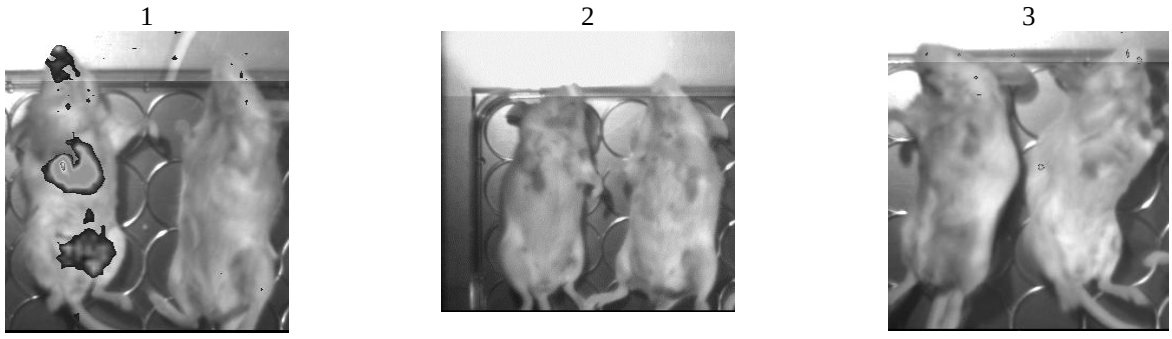
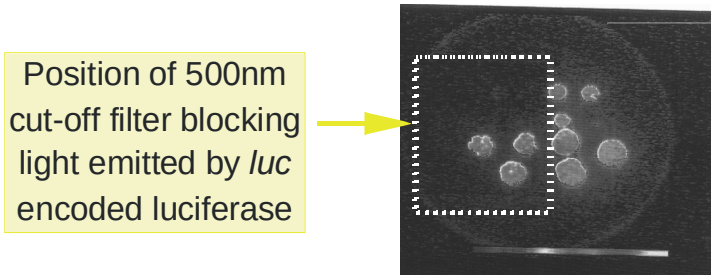


Figure 4. Differentiation of bacteria expressing *luc* (upper half of the plate) and *lux* genes (lower half of the plate).



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