Use of Bioluminescent and Fluorescent Bacteria to Study Food-Borne Pathogens

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Introduction

The construction of bioluminescent and fluorescent food-borne pathogens to study behavior in food systems is an expanding area of research. Using recombinant DNA technology, food microbiologists can create bioluminescent pathogens that enable the study of growth, injury, and destruction in complex systems. In addition, bioluminescence can be employed in rapid detection of pathogens through the use of genetically engineered bacteriophage. Fluorescent pathogens can be constructed to study the spatial distribution and behavior of bacteria in solid food systems. This update session will be a brief overview of these exceptional research tools and applications in food microbiology.

Bioluminescence

Any living organism that emits light is considered to be bioluminescent. Four genera of bacteria (*Vibrio, Photobacterium, Alteromonas,* and *Xenorhabdus*) naturally fall under this category (Baker et al., 1992). All bacterial systems are related and consist of a luciferase enzyme that binds to cellular reduced flavin mononucleotide ($FMNH_2$) in the presence of oxygen to form an intermediate peroxide. This intermediate then reacts with long chain aldehydes to produce oxidized flavin mononucleotide, fatty acids, and the release of blue green light (Hastings, 1996). Genetically, bacterial luciferase systems are made up of a regulatory region, genetic determinants encoding the two subunits of enzyme luciferase, and a set of genes required for synthesis of aldehydes from fatty acids (Baker et al., 1992). When genetically engineering "dark" bacteria to be luminescent, there are two options: 1) introduce all genetic material needed to synthesize both the luciferease and aldehydes for generation of light; or 2) introduce only the genetic material encoding the luciferase enzyme and add additional adehyde to the system for light production (Baker et al., 1992; Hill & Stewart, 1994). The latter strategy is most common using a gene fusion of both the luciferase genes from *Vibrio harveyi* (*luxAB,* often called the Lux system) (Chen & Griffiths, 1996; Duffy et al., 1995; Jacobs et al., 1995; Loessner et al., 1996; Loessner et al. , 1997). The amount of light released is a function of the amount of $FMNH_2$ available in the bacterial cells. The advantages of using bioluminescent bacteria or bacteriophage are the sensitivity (detect as few as 100-500 bacteria), speed (less than 1 h), and high correlation between cell number and bioluminescence (Baker et al., 1992; Chen et al., 1996).

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Bioluminescent Bacteria to Study Injury and Survival of Pathogens

Genetically engineered bioluminescent pathogens can be used to track the injury and survival of pathogens in laboratory model systems. Food processing (heat treatments, freezing, changes in acidity or water activity) will induce stress and adaptive responses in bacterial cells that will directly or indirectly influence the level of cellular $FMNH_2$. Since luminescence is directly proportional to $FMNH_2$, the Lux system is ideal for studying sub-lethal injury and cellular adaptation (Duffy et al., 1995). Researchers have found a high correlation between bioluminescence and plate count data thermal inactivation in injury studies using *Salmonella typhimurium* (*S. typhimurium*) (Duffy et al., 1995).

Foods almost always have a mixed population of bacteria, yet food microbiologists have traditionally used pure cultures for experiments. An advantage of using a pathogen expressing a Lux system is that it allows for ease of detection of a target organism in the presence of high populations of endogenous microflora or bacterial starter cultures. Use of bioluminescent strains can overcome difficulties encountered with recovery of injured organisms by selective/differential plating, as the light emitted by the recombinant strain can be easily separated from the background microflora. Since light measurement is more sensitive than plate counts, low levels bioluminescent pathogens can be detected in the presence of high levels of competitive microflora (Duffy et al., 1995).

Using a bioluminescent *S. typhimurium* strain, Duffy et al. (1995) found that high levels of competitive microflora (1 x 10^8 CFU/ml) increased the decimal reduction (D-value) at 55°C to 2.09 min from 0.43 min with pure culture (Duffy et al., 1995). This is a dramatic effect that would not be observed using pure cultures for thermal destruction experiments. Other researchers have used bioluminescent strains in to monitor *E. coli* O157:H7 survival in model fermented sau-

sage systems (Tomicka et al., 1997) and evaluate the level sub-lethal injury of *Salmonella enteritidis* due to reduced pH and heat processing (Chen & Griffiths, 1996).

Bioluminescence Bacteriophage for Rapid Detection of Food-borne Pathogens

Researchers have inserted bioluminescence determinants into bacteriophage for use as rapid bacterial detection systems. In these systems, the *luxAB* genes are genetically introduced into bacteriophage and engineered phage are used to detect bacteria in the sample. When the target bacteria are present in a sample, the phage particles adsorb to the cells and inject the DNA containing the *luxAB* luciferase gene. As the phage genome is transcribed and translated, luciferase accumulates in the cell. Aldehyde is added to the system and light is measured in a luminometer. Since bacteriophage only replicate in living cells, a linear relationship exists between viable cell numbers and bioluminescence (Baker et al., 1992). The detection of viable bacteria is unique to this method, since other methods such as PCR or ELISA have the potential to detect both viable and non-viable cells.

Like most rapid detection systems, detection by phage bioluminescence requires enrichment of the food sample to accumulate sufficient cell numbers for detection, but after enrichment this test is rapid (less than 1 hour), detects only viable cells, and requires very little technical training. In addition, the reagent costs for a bacteriophage based test are relatively inexpensive due to the ease of preparation of phage stocks. The greatest hindrance to the development of these tests is the dependence on the specificity of the bacteriophage. For example, to detect a genus such as *Salmonella* you need a phage that will infect all the *Salmonella* species. If the phage specificity is too narrow (binds a fraction of all *Salmonella* types), false negative results could occur. In the same manner, if you wanted to detect a specific serotype of bacterium, such as *E. coli* O157:H7, phage specificity should be very narrow to prevent false positive results. Thus, the infectious range of the bacteriophage will determine the degree of false positive or false negative results by a bioluminescent phage detection method.

Bioluminescent bacteriophage systems have been developed to detect *Salmonella typhimurium* and *Listeria monocytogenes* (*L. monocytogenes*) using bacteriophage that has been well characterized over years of research (Loessner et al., 1996; Loessner et al., 1997; Stewart et al., 1989; Turpin et al., 1993). P22 phage is specific for only *Salmonella typhimurimum*. The *luxAB* fusion has been cloned into the P22 genome and used to detect of as few as 100 cells in 50 minutes (Stewart et al., 1989). Detection of *Listeria monocytogenes* (*L. monocytogenes*) has been performed with a phage A511 with a *luxAB* fusion added to the major capsid protein added (Loessner et al., 1996; Loessner et al., 1997). This myovirus infects 95% of *L. monocytogenes* serovar 1/2 and 4 cells. Researchers have found listerial detection similar to traditional methods (1-10 cells in 25g) after a 20 hour enrichment. Different foods have been shown to influence the sensitivity and the amount of detectable luminescence (Loessner et al., 1997). Bioluminescent phage can be used in a modified MPN technique to quantify low levels of target bacteria environmental samples or foods (Loessner et al., 1997; Turpin et al., 1993).

Use of Fluorescent Strains in Food Microbiology

Another approach to modeling bacteria in foods is to construct fluorescent strains that express green fluorescent protein (GFP). This protein, originally isolated from the jellyfish *Aequorea victoia,* does not require any unique factors to fold into the fluorescent structure (Patterson et al., 1997). Therefore, one genetic determinant can be transferred into bacteria, and in the presence of oxygen, GFP is formed intracellularly (Patterson et al., 1997). This system produces fluorescent strains without any additional substrate or stain. The original GFP has been modified by a number of researchers to alter its fluorescent emission spectra and decrease sensitivity to photobleaching (Patterson et al., 1997). One application of GFP expressing strains its use as a marker to monitor bacterial survival. GFP expressing *E. coli* O157:H7 survival in apple cider and orange juice during refrigerated storage were monitored by shining a UV light on the petri plates to differentiate this pathogen from background bacteria (Fratamico et al., 1997).

Another application of GFP expressing strains is to study the spatial distribution and bacterial adherence in solid foods using laser scanning confocal microscopy (LSCM). LSCM uses laser illumination that can be focused at a specific depth within a solid sample allowing for microscopic observation and three dimensional analysis of solid foods. Since GFP expression has no influence on bacterial surface characteristics important for bacterial adherence, GFP expressing strains are ideal for observing bacterial adherence or behavior in biofilms.

Conclusions

Bioluminescence and fluorescence are tools that will become more common in food microbiology in the future. The sensitivity, speed and flexibility of these systems for monitoring bacteria will allow for more unique ways to study of pathogens in complex food systems.

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