

**United States Patent**

**Goodridge**

**Template Reporter Bacteriophage Platform and Multiple Bacterial Detection Assays Based Thereon**

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Field of Classification Search

None

See application file for complete search history.

References Cited

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**ABSTRACT**

The invention is a method for the development of assays for the simultaneous detection of multiple bacteria. A bacteria of interest is selected. A host bacteria containing plasmid DNA from a T even bacteriophage that infects the bacteria of interest is infected with T4 reporter bacteriophage. After infection, the progeny bacteriophage are plating onto the bacteria of interest. The invention also includes single-tube, fast and sensitive assays which utilize the novel method.

6 Claims, 3 Drawing Sheets


* cited by examiner
**FIG. 1**

Plasmid

**T4 flanking DNA**

**p22/lacZ fusion**

**T4 phage chromosome**

**in vivo recombination**

**T4 flanking DNA**

**T4 flanking DNA**

**T4 lacZ reporter phage**

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Bacteriophage with required host range

Gene 36
Genes 37 and 38
Gene t

Plasmid

Genes 37 and 38

in vivo recombination

Gene 36
Genes 37- and 38-
Gene t

T4 phage chromosome

T4 lacZ reporter phage

FIG. 2
FIG. 3

TABLE

<table>
<thead>
<tr>
<th>Initial Inoculum (CFU/100 cm²)</th>
<th>Average RLU</th>
<th>Detection Threshold (3x Std dev)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 E2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 E1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 E0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

FIG. 4
1

TEMPLATE REPORTER BACTERIOPHAGE PLATFORM AND MULTIPLE BACTERIAL DETECTION ASSAYS BASED THEREON

CLAIM OF PRIORITY


STATEMENT OF GOVERNMENTAL INTEREST

The United States Government has rights in this application based on NASA Space Consortium Grant No. NCC5-578.

BACKGROUND OF THE INVENTION

The invention relates generally to platforms for the development of assays for the simultaneous detection of multiple bacteria and, more specifically, the use of the T4 bacteriophage, and, more specifically, the use of the T4 bacteriophage, non-viable bacteria. This is a major advantage over conventional methods used to create the genetically modified bacteriophage. This method described in this invention, in which multiple assays can be produced from a single bacteriophage, is advantageous and cost effective because the assays can be produced based on a standardized platform, without the need to completely genetically and phenotypically characterize new bacteriophage for every new test.

One disadvantage of the currently available reporter bacteriophage assays is the need for an instrument to detect the reporter protein of interest, which limits the ability of these methods to be used in the field. The present invention also includes a single-tube apparatus that employs the reporter bacteriophage platform in an assay that is simple and easy to perform, inexpensive and fast. This reporter bacteriophage assay is self-enclosed, and detected using a choice of substrates. One class of these substrates (colorimetric substrates) allows for the production of a visible colorimetric product, eliminating the need for instrument based detection.

SUMMARY OF THE INVENTION

The invention consists of a method for the simultaneous development of multiple bacterial detection assays based on a T4 bacteriophage system. A T4 reporter bacteriophage strain is used that carries several genetic mutations. First, the T4 reporter bacteriophage strain carries amber mutations in the genes denA and denB. As a result of the amber mutations in denA and denB, the host DNA and plasmid DNA will remain intact within the bacterial cell upon infection with the T4 reporter strain. The T4 reporter bacteriophage strain has also been genetically modified to carry a reporter gene. In a preferred embodiment, the reporter gene is a thermophilic β-galactosidase gene (lacZ) that has been fused, in frame, to the T4 promoter 22. The promoter 22 is stably integrated in a non-essential part of the T4 genome. The T4 reporter bacteriophage also has amber mutations in its tail genes 37 and 38 so that the T4 reporter bacteriophage is only capable of growth in a bacterial host strain that suppresses amber mutations. In addition, the T4 reporter gene will only be capable of growth when mutated tail genes 37 and 38 are replaced with functional genes via marker rescue.

This invention also consists of the development of an assay for the rapid detection of pathogenic bacteria. Such an assay is capable of producing accurate results from complicated samples containing mixtures of microorganisms. This invention will address the limitations of the currently available methods for detection of bacterial pathogens through the development of a one-tube system that will be capable of rapidly detecting viable pathogenic isolates. The detection system consists of two components, including a reporter bacteriophage genetically modified to carry a thermophilic β-galactosidase gene, and a substrate for the β-galactosidase, which is encapsulated within temperature sensitive liposomes. The substrate consists of any substrate that can be used for detection of β-galactosidase, including the colorimetric, fluorescent and luminescent substrates.

The release of drugs/molecules in an environment controlled by the use of temperature-sensitive liposomes is a
novel approach. Liposomes are phospholipid vesicles and work as carriers for the delivery of molecules to cells. For example, temperature sensitive liposomes have been designed to release drugs in response to increased temperatures at the disease site. In this invention, the β-galactosidase substrates are encapsulated within temperature sensitive liposomes, which are designed to release the substrate upon an increase in temperature. The liposomes are designed so that they can be specifically targeted to the surface of the bacterial cell of interest. This can be achieved by incorporating specific antibodies, or other targeting ligands onto the surface of the liposomes, so that they will attach to a given bacteria in a specific manner. Targeting the liposomes to the bacterial cell surface will result in intimate contact between the enzyme and substrate, allowing for an increase in sensitivity of the assay.

In a preferred embodiment, the assay is incorporated into a single tube, such as the Snap-Valve device sold by Medical Packaging Corporation, or any other single tube device that is designed to be used, or can be used, in conjunction with a hand held luminometer.

**BRIEF DESCRIPTION OF THE FIGURES**

**FIG. 1** is a diagrammatic representation of the construction of a LacZ T4 reporter phage; the p22/LacZ fusion is cloned into a plasmid, and is flanked by the T4 recombinational genetic targets; the p22/LacZ fusion integrates into the bacteriophage T4 chromosome via a double homologous recombination event between the plasmid-borne target sequences and their bacteriophage chromosomal counterparts.

**FIG. 2** is a diagrammatic representation of the construction of a LacZ T4 reporter phage with altered host range; the T4 phage with an integrated lacZ gene of FIG. 1, and amber mutations in tail genes 37 and 38 (37T, 38T), is used to create a reporter phage with an altered host range.

**FIG. 3** is a diagrammatic representation of a single compartment assay scheme of the present invention.

**FIG. 4** is a chart of luminescent detection with an assay apparatus of the present invention; the average reading represents the mean of three readings per sample; standard deviations are also indicated; the assay was capable of detecting an initial inoculum of 10^4 CFU/100 cm^2.

**DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS**

**Example 1**

In general, the method uses a T4 reporter bacteriophage with genetic mutations to detect various bacterial pathogens. A T4 reporter bacteriophage strain is used that carries several genetic mutations. First, the T4 reporter strain carries amber mutations in the genes denA and denB. These non-essential genes are responsible for degrading bacterial host and plasmid DNA upon infection of the host bacterial cell. As a result of the amber mutations in denA and denB, the host DNA and, most importantly, plasmid DNA remain intact within the bacterial cell upon infection with the T4 reporter strain. This results in much higher frequencies of homologous recombination between plasmid borne targets and the bacteriophage chromosome.

As shown in FIG. 1, the T4 reporter bacteriophage strain has also been genetically modified to carry a reporter gene. The reporter gene is a β-galactosidase gene (LacZ) which has been fused, in frame, to the T4 promoter 22. The promoter 22/β-galactosidase fusion is stably integrated in a non-essential part of the T4 genome.

Finally, the T4 reporter bacteriophage has amber mutations in its tail genes 37 and 38. As a result of these amber mutations in the tail genes, the T4 reporter bacteriophage is only capable of growth in a bacterial host strain that suppresses amber mutations, such as E. coli CR63 F^+, supD60. Using an amber suppressing strain such as CR63 amplifies the T4 reporter bacteriophage. In addition, the T4 reporter gene is only capable of growth when mutated tail genes 37 and 38 are replaced with functional genes via marker rescue. This T4 reporter bacteriophage is used in detection assays to detect bacterial pathogens in the following manner. First, a T even bacteriophage that infects the bacteria of interest is amplified by PCR using the above-described primers and cloned into a suitable plasmid. The plasmid is placed into the amber suppressing bacterial host strain (CR63). The T4 reporter bacteriophage, which includes the amber mutations in tail fiber genes 36 and 37, is used to infect the amber suppressing bacterial host strain (CR63).

As shown in FIG. 2, once the T4 reporter bacteriophage introduces its DNA to the host bacteria, its DNA recombines with the plasmid containing the host range cassette. The plasmid borne host range cassette integrates itself into the T4 reporter bacteriophage chromosome via double homologous recombination (marker rescue). Bacteriophage that have functional tail fiber genes as a result of the marker rescue step are isolated by plating the bacteriophage on the bacterial host of interest, which supports the growth of the bacteriophage that have received the functional tail fiber genes, but cannot support the growth of the amber mutant bacteriophage.

For example, in the development of a detection assay for *Salmonella*, the host range cassette from a T even bacteriophage that infects *Salmonella* is amplified, cloned into a plasmid, the plasmid is placed into the *E. coli* amber suppressing strain (CR63), the strain is infected with the template reporter phage, progeny phage are isolated, and bacteriophage are grown on a *Salmonella* strain. The *Salmonella* strain does not allow for the template reporter phage with the amber mutations in genes 37 and 38 to grow (because it does not have the right host range, and wild type strains do not usually carry suppressors for amber mutations), but only allows bacteriophage that have functional tail fiber genes (obtained from the *Salmonella* T even bacteriophage, by marker rescue) to grow.

This provides an effective system for the rapid creation of detection assays that specifically detect different bacterial...
pathogens, and are all based upon the same platform, which in the preferred embodiment is transduction of a thermophilic β-galactosidase gene.

Example II
This invention consists of the development of an assay for the rapid detection of pathogenic bacteria. Such an assay is capable of producing accurate results from complicated samples containing mixtures of microorganisms. This invention addresses the limitations of the currently available methods for detection of bacterial pathogens through the development of a one-tube system that is capable of rapidly detecting viable pathogenic isolates. The detection system consists of two components, including a reporter bacteriophage genetically modified to carry a thermophilic β-galactosidase gene, and a substrate for the β-galactosidase, which is encapsulated within temperature sensitive liposomes. The substrate consists of any substance that can be used for detection of β-galactosidase, including the colorimetric, fluorescent and luminoigenic substrates.

The release of drugs/molecules in an environment controlled by the use of temperature-sensitive liposomes is a novel approach. Liposomes are phospholipid vesicles and bacteriophage, 0-galactosidase substrate, bacterial growth controlled by the use of temperature-sensitive liposomes is a dippers, and drippers. The device containing the reporter bacteriophage, 0-galactosidase gene.

The rapid detection of pathogenic bacteria. Such an assay is capable of detecting any viable bacteria of interest within the sample, and force the bacteria to make large amounts of 0-galactosidase and following the 45 interact with the 0-galactosidase, causing a visual colorimetric reaction. The signal is detected by a calorimetric substrate, or require the use of instrumentation to observe the signal (as when fluorescent or luminoigenic substrates are used). The nature of this detection method is such that it is effective at sensitively and specifically detecting viable bacteria. Also, the assay is rapid, and easy to perform. Assays for multiple pathogens may be included within a single kit. For example, a kit for the simultaneous detection of E. coli O157, Salmonella, and Shigella is comprised of three individual kits, all of which are identical except that the reporter bacteriophage used for detection have different host ranges (one each for E. coli, Salmonella, and Shigella). The tests are performed simultaneously, wherein is a single sample that is divided into three sections. Each section is placed into an individual tube, and the assay is performed. In this way simultaneous and specific detection of multiple pathogens is effected.

Example III
An improved, self-contained single tube apparatus for carrying out the assays of the present invention has been developed. The reporter bacteriophage and the substrate (any colorimetric substrate) are now included within a Snap-Valve™ device or any variation of the Snap Valve™ device (Medical Packaging Corporation, Calif.). The Snap-Valve™ device also houses growth media for the bacteria, immunomagnetic separation beads specific for the target bacteria and the sampling device which consists of any of the following: swabs, scrapers, scoopers, loops, needles, honey dippers, and drippers. The device containing the reporter bacteriophage, β-galactosidase substrate, bacterial growth media, immunomagnetic separation beads, and the sampling device has been termed the Phast Swab. To test the food sample in question or the food contact surface, or an environmental surface (such as a drain), or any other sample, the sampling device is removed from the Snap Valve™ device and a sample is obtained. The sampling device is replaced into the Snap Valve™ and the entire device is incubated at 31°C to 37°C for 8 hours. Following the incubation step, immunomagnetic separation (IMS) is accomplished on the sample by placing the entire device into a magnetic concentrator, allowing the IMS beads to concentrate, and removing the growth media. Next, the bottom compartment of the Snap Valve™ is punctured, releasing the reporter bacteriophage to mix with the beads. The Snap Valve™ is incubated at 31°C to 37°C for 1.5 hours, during which the reporter bacteriophage will infect any target cells present, and force them to make β-galactosidase. Then, the cap of the Snap Valve™ is broken, releasing the substrate to mix with the reporter bacteriophage, IMS-bacteria suspension. If β-galactosidase was produced, then the substrate will interact with the β-galactosidase, causing a visual colorimetric reaction, indicating a positive result. A negative test is indicated by the absence of a visual colorimetric reaction.

The system has been modified as described in more detail below to be read in portable hand held luminometers.

To demonstrate the effectiveness of the present assay apparatus, ten by ten centimeter squared portions of beef (top round, sirloin tip) were inoculated with serial dilutions of E. coli O157:H7 by pipetting 1 ml of each dilution on an individual 10x10 cm² piece of meat, followed by spreading the dilution over the entire surface of the meat with a glass hockey stick. The meat was allowed to dry for one hour, and then the entire surface of each meat sample was swabbed with an individual Snap-Valve™ device. The colorimetric swab assay was performed as follows: in the colorimetric swab assay, the swab was removed, the surface of the meat was swabbed and the swab was returned to the Snap-Valve™ device, followed by an 8 hour enrichment. Following enrichment, the IMS beads (with E. coli O157 cells attached) were concentrated, and the growth media was removed. Following a wash step, the reporter phage was added (10⁷ PFU/ml), and the Snap-Valve™ device was incubated at 37°C for 1.5 hours. Finally, the cap of the
Snap-Valve™ device was broken, releasing the β-galactosidase (CPRG) substrate into the bottom of the device, where it reacted with any β-galactosidase present. A positive test was indicated by the development of a red color, while in a negative test, the color remained yellow. Two negative controls were included in the assay. They consisted of a cell only control, which contained E. coli O157:H7 but no reporter phage, and a phage only control that contained the reporter phage, but no E. coli O157:H7 bacteria. The results indicated that the assay apparatus of the present invention could detect an initial inoculum of 10^2 CFU/100 cm² of meat.

To test the ability of the assay apparatus of the present invention to detect E. coli O157:H7 in a more sensitive fashion, the above experiment was repeated, except that in this case the β-galactosidase substrate consisted of a luminescent substrate. At the end of the assay, the Snap-Valve™ device was placed into a portable hand held luminometer, and the results were read (FIG. 4). Using the luminescent substrate, the assay apparatus of the present invention was able to detect an initial concentration of 10^1 CFU/100 cm² of meat.

The foregoing description and drawings comprise illustrative embodiments of the present inventions. The foregoing embodiments and the methods described herein may vary based on the ability, experience, and preference of those skilled in the art. Merely listing the steps of the method in a certain order does not constitute any limitation on the order of the steps of the method. The foregoing description and drawings merely explain and illustrate the invention, and the invention is not limited thereto, except insofar as the claims are so limited. Those skilled in the art who have the disclosure before them will be able to make modifications and variations therein without departing from the scope of the invention.

I claim:

1. An assay kit for detecting the presence of viable target bacteria in a sample, comprising:
   (a) a container for the sample;
   (b) media in the container which supports growth of the target bacteria;
   (c) specific separation beads in the container to concentrate the target bacteria;
   (d) a sampling device within the container to rapidly obtain the sample to be tested;
   (e) a lytic reporter phage in the container capable of infecting a viable target bacterium in the sample which upon incubation produces the reporter; and
   (f) a detector substrate released into the media which produces a signal in the presence of the reporter.

2. The assay kit as defined in claim 1, wherein the detector substrate is contained in a capsule that releases the substrate upon heating of the media to an elevated temperature following incubation of the sample.

3. The assay kit as defined in claim 2, wherein the capsule is a liposome.

4. The assay kit as defined in claim 1, wherein the phage contains a DNA sequence derived from the host range cassette of the target bacteria.

5. The assay kit as defined in claim 1, wherein the reporter is β-galactosidase.

6. The assay kit as defined in claim 1, wherein the lytic phage is a T4 phage.