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(54) Title: COMPOSITIONS AND METHODS FOR BACTERIA DETECTION

(57) Abstract: Phage including one or more detectable nucleotide sequences are described. In addition, methods of using the phage to detect viable bacteria are described.

COMPOSITIONS AND METHODS FOR BACTERIA DETECTION

FIELD OF THE INVENTION

This invention relates to an assay method for quickly and easily detecting the presence of bacteria. In particular, this invention is directed to phage compositions and methods of using phage to detect bacteria.

BACKGROUND OF THE INVENTION

The presence of bacterial pathogens is a well recognized cause of severe illness. As such, there is an ever present need for the detection of such pathogens in both clinical specimens (i.e. blood, tissue, urine and other body extracts and fluids), agricultural specimens (such as food or feed products) and environmental specimens (such as surfaces in food processing plants, contact lens solution, or water samples).

Current tests for the detection of bacterial pathogens, such as in food, typically require a number of days to complete. During this period of time, between sampling and assay determination, fresh food and dairy products will enter the food chain and therefore be consumed by the public. If a test indicates the presence of pathogens, expensive product recalls may result, or, worse, before the test results are known an outbreak of sickness may occur. The very rapid immuno-based tests tend to lack sensitivity, specificity and cannot determine pathogen viability. In some applications immuno-based tests require pre-culture for 18-36 hours.

As stated above, traditional methods to detect the presence of bacterial food pathogens require an extended period of time, basically due to the need for an enrichment/incubation period. This incubation/enrichment period is intended to allow for growth of these bacteria from a background of competing microorganisms and an increase in bacterial cell numbers to more readily aid in identification. In many cases

a series of two or three separate incubations is needed to isolate the target bacteria.

However, such enrichment steps can actually compromise test sensitivity by killing some of the cells sought to be measured. Overgrowth by background flora is always a concern.

Bacteriophages (commonly called phages) are bacteria infecting viruses. They usually display host specificity. During a course of infection, bacteriophages gain access to the host bacterium via bacterial cell surface constituents (referred to as receptors) through specific recognition and attachment interactions between these receptors and bacteriophage particle surface ligands.

The concept of using bacteriophages to type host bacteria is commonly practiced in the art although such a typing method is typically utilized to further complement serotyping and other pathogenic typing methods. Many authors have disclosed how the specificity of bacteriophages may be used to distinguish between bacterial genus, species or serotype (serovar). In J. Clin. Microbiol. 20 (1984) 1122-1125, Cooper et al. disclose the use of bacteriophages to distinguish between certain species of Bacteroides. Van der Walt and Stein disclose how *Salmonella* and *Citrobacter* sub-species may be differentiated using the specificity of bacteriophages [Onderstepoort J. Vet. Res. 56 (1989) 263-269). He and Pan, in J. Clin. Microbiol. 30 (1992) 590-594, disclose how bacteriophages may be used to distinguish between types of *Enterobacteria* present in clinical specimens, and Liew and Alvarez demonstrated that subtypes *of Xanthomonas campestris* can be distinguished using the specificity of bacteriophages [Phytopathol. 71 (1981) 274-276].

Detection of specific bacteria via genetically engineered bioluminescent bacteriophages which have had the 'lux' gene inserted into their genome has also been described [Ulitzer and Kuhn, in Scholmerich *et al* (Eds) "Bioluminescence and chemiluminescence - new perspectives", pages 463-472: published in 1987 by John

Wiley and Sons]. This technique is based on the fact that upon infection of a target bacterium, bacteriophage genes and the 'lux' gene are injected into the host bacterium and are subsequently expressed. The presence of a target bacterium is indicated by emission of light generated from the activity of the 'lux' gene that can be measured. While useful, this technique requires special equipment to measure light emission and often does not provided the necessary detection sensitivity.

There is thus a widely recognized need for a bacteriophage mediated bacterial typing method devoid of the above mentioned limitations. Specifically, there is a widely recognized need for a bacteriophage mediated bacterial typing method for quickly confirming and semi-quantitating the presence of viable bacteria, including potentially pathogenic bacteria.

SUMMARY OF THE INVENTION

It is, therefore, an object of the present invention to provide compositions and methods for the detection and identification of viable bacteria. It is another object of the invention to provide a method for quickly confirming and semi-quantitating the presence of any viable bacteria, including potentially pathogenic bacteria.

In order to meet these needs, the present invention is directed to compositions and methods of using species/strain specific bacteriophages as indicators of viable microbial contamination in various sample types.

Virtually all bacteria have infecting phages that are species specific. The phage of the invention are genetically modified by recombinant DNA techniques to include a signature or identifying DNA or RNA sequence or product produced from these sequences which is not normally present in wild type populations of bacteria or other phage. The present invention is directed to compositions comprising modified phage. The modified phage may be packaged in the form of a kit. The modified phage are useful to rule out any possible wildtype phage that maybe present in the

sample. The modified phage of the invention may be used alone or in combination with various other modified phage.

The modified phage of the invention are utilized to detect bacteria in a sample. Samples may be from any source. Sample sources include clinical specimens, agricultural specimens and environmental specimens. The modified phage of the invention are combined with the sample under suitable conditions and for a suitable time period to permit the modified phage to locate and infect a suitable, viable host, if present. If such a viable host is present in the sample, the modified phage will infect the host and proceed to replicate itself many fold and produce large amounts of the reporter molecule.

After a suitable host infection and replication time period, the sample is analyzed for the presence of the phage of the invention or products produced from it.

The present invention is also directed to methods of detecting the modified phage of the invention in order to determine the presence of viable bacteria in the sample. The methods of the invention do not detect dead bacteria because the modified phage of the invention, like all phage, infect and multiply only in viable bacteria. Dead bacterial cells are not susceptible to phage infection and replication.

The modified phage of the invention include a signature DNA or RNA repeat sequence. The signature DNA or RNA sequence may be detected by any of a number of procedures known to one of skill in the art. For example, detection can be by amplification (i.e. PCR, LCR, NASBA, etc.) of the repeat sequence, followed by gel electrophoresis or by direct hybridization of a fluorescent or non-fluorescent probe to an amplicon and analysis. Detection may also occur without the need of any additional amplification processes since the phage may amplify the target sequence up to high enough levels where it can easily be detected directly (i.e. probe hybridization techniques).

Depending on the phage replication method and detection protocol, the target repeat sequence may be silent or absent in phages that have not undergone replication and activated or produced in those which have infected a cell and replicated. Alternatively a protein may be produced only when infection has occurred. When there is phage infection, the levels of phage rise above the background levels of a non-infection. This increase over background levels can also be detected. As such, the probe is detectable upon phage replication.

The present invention is thus directed to a method of detecting a viable bacterial host for a phage in a sample. The method of the invention includes a) combining a modified phage with a sample wherein the modified phage includes one or more detectable nucleotide sequences to form a modified phage sample mixture; b) incubating the modified phage sample mixture under suitable conditions to permit the modified phage to infect a bacterial host for the modified phage and amplify in the bacterial host to form amplified modified phage and c) detecting the nucleotide sequence in the amplified modified phage to thereby detect a viable host for said phage.

The present invention is further directed to isolated oligonucleotides or DNA for use in the invention. The isolated oligonucleotides or DNA have a sequence which is not present in either phage or bacterial DNA and thus permit specific detection of viable bacteria as described above. The oligonucleotide or DNA will generally be 20 to 30 nucleotides in length, but can be 100 or more nucleotides in length.

The present invention is further directed to recombinant phage DNA wherein the phage DNA includes the oligonucleotide and/or the DNA of the invention. The present invention is further directed to phage wherein the phage includes the oligonucleotide or DNA of the invention.

The present invention is further direct to kits including the recombinant phage DNA of the invention. The recombinant phage DNA may be present in phage.

DETAILED DESCRIPTION OF THE INVENTION

As noted above, one object of the invention is to provide phage compositions and assay procedures for rapid and easy identification of the presence of a particular bacteria of interest in a sample. The phage compositions and assay procedures are broadly applicable to the detection of any bacteria.

In a particular preferred aspect, the assay is used to detect various bacteria, and can be utilized to detect the presence of any specific, selected bacteria of interest for which a phage exists. The bacteria can be either pathogenic or non-pathogenic, although the invention is particularly important for detection of potentially contaminating pathogenic bacteria. Specific bacteria detectable by the assay of the invention include, for example, *Listeria*, *Campylobacter*, *Escherichia coli*, *Salmonella*, *Clostridia* (such as *Clostridium botulinum* and *Clostridium perfingens*), *Shigella*, *Staphylococci* (such as *Stapylococcus aureus*), *Vibrio* (such as *Vibrio vulnificus*, *Vibrio cholerae* and *Vibrio parahaemolyticus*), *Yersinia* (such as *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*) *Plesiomonas strigelloides*, *Bacilli* (such as *Bacillus cereus*) *Aeromonas* (such as *Aeromonas hydrophila*), *Methicillin-resistant S. aureus (MRSA), Staph epidermidis, Salmonella typhi, Salmonella typhimurium, Shigella dysenteriae, Listeria monocytogenes, Corynebacterium diphtheriae, Chlamydia, Streptococcus pneumoniae, Streptococcus pyogenes, Strep agalactiae, Group A Streptococcus (GAS), Bacillus anthracis, Mycobacterium tuberculosis, M. avium, M. leprae, M. bovis, Rickettsia rickettsii, Ehrlichia chaffeensis, Borrelia burgdorferi, Yersinia pestis, Yersinia enterocolitica, Treponema pallidum, T. carateum, Trichomonas vaginalis, Neisseria gonorrhoeae, Neisseria meningitides, Enterococcus faecalis, Enterococcus faecium,, Klebsiella pneumoniae,*

Serratia marcescens, Enterobacter cloacae, Enterobacter aerogenes, Haemophilus influenzae, Haemophilus parainfluenzae, Legionella pneumophila, Legionella dumoffii, Pseudomonas aeruginosa, Clostridium tetani, Clostridium botulinum, Clostridium difficile, Clostridium perfringens, Bateroides fragilis, Mycoplasma pneumoniae, Mycoplasma hominis, Chlamydiae pneumoniae, Chlamydiae trachomatis, Chlamydiae psittaci, Helicobacter pylor, Campylobacter jejuni, Campylobacter coli, Bordetella pertussis, Borrelia burgdorferi, and indicator groups of bacteria such as Enterococci. The invention may also be used to detect sub-species of bacteria such as *E. coli O157:H7.*

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not limited in its application to the details of construction and the arrangement of the components set forth in the following description. The invention is capable of other embodiments or of being practiced or carried out in various ways. Also, it is to be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting.

Throughout this disclosure, various publications, patents and published patent specifications are referenced. The disclosures of these publications, and published patent specifications are hereby incorporated by reference into the present disclosure to more fully describe the state of the art to which this invention pertains.

Definitions

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of immunology, molecular biology, microbiology, cell biology and recombinant DNA technology, which are within the skill of the art. See, e.g., Sambrook, Fritsch and Maniatis, MOLECULAR CLONING: A LABORATORY MANUAL, 2nd edition (1989); CURRENT PROTOCOLS IN MOLECULAR BIOLOGY

(F. M. Ausubel, et al. eds., (1987)); the series METHODS IN ENZYMOLOGY (Academic Press, Inc.): PCR 2: A PRACTICAL APPROACH (M.J. MacPherson, B.D. Hames and G.R. Taylor eds. (1995)), Harlow and Lane, eds. (1988) ANTIBODIES, A LABORATORY MANUAL, and ANIMAL CELL CULTURE (R.I. Freshney, ed. (1987)).

The term "polynucleotide", "oligonucleotide", or "nucleic acid" refers to a polymeric form of nucleotides of any length, either deoxyribonucleotides or ribonucleotides, or analogs thereof. The terms "polynucleotide" and "nucleotide" as used herein are used interchangeably. Polynucleotides may have any three-dimensional structure, and may perform any function, known or unknown. The following are non-limiting examples of polynucleotides: a gene or gene fragment, exons, introns, messenger RNA (mRNA), transfer RNA, ribosomal RNA, ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs. If present, modifications to the nucleotide structure may be imparted before or after assembly of the polymer. The sequence of nucleotides may be interrupted by non-nucleotide components. A polynucleotide may be further modified after polymerization, such as by conjugation with a labeling component. A "fragment" or "segment" of a nucleic acid is a small piece of that nucleic acid.

A "gene" refers to a polynucleotide containing at least one open reading frame that is capable of encoding a particular protein after being transcribed and translated.

The terms "primer" and "nucleic acid primer" are used interchangeably herein. A "primer" refers to a short polynucleotide, whether occurring naturally as in a purified restriction digest or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a

primer extension product, which is complementary to a nucleic acid strand, is induced, i.e., in the presence of nucleotides and an inducing agent such as a polymerase and at a suitable temperature and pH. The primer may be either single-stranded or double-stranded and must be sufficiently long to prime the synthesis of the desired extension product. The exact length of the primer will depend upon many factors, including temperature, source of primer and use of the method.

A "polymerase chain reaction" ("PCR") is a reaction in which replicate copies are made of a target polynucleotide using a "primer pair" or a "set of primers" consisting of an "forward" and a "reverse" primer, and a catalyst of polymerization, such as a DNA polymerase, and particularly a thermally stable polymerase enzyme. Methods for PCR are taught in U.S. Patent Nos. 4,683,195 (Mullis) and 4,683,202 (Mullis et al.). All processes of producing replicate copies of the same polynucleotide, such as PCR, TMA, LCR (etc.) or gene cloning, are collectively referred to herein as "amplification" or "replication".

As used herein, the term "type" when used in context of bacteria is meant to include either a genus, a species and a subspecies e.g., it strain and/or a serovar. As such bacteria of a certain type can be bacteria of a certain genus, species or subspecies (strain or serovar). When used in context of bacteriophages, the term "type" refers to the host specificity of that bacteriophage, i.e., bacteriophages of different types have somewhat different bacterial type specificity.

The terms "serovar" and "serotype" are used herein interchangeably and refer to a bacterial isolate which is reactive to specific antiserum prepared against this isolate (polyclonal, monospecific or monoclonal). As such, a serovar displays unique antigenic determinant(s), some or all of which may or may not be present on other isolates of a strain or on other strains of a species.

The terms "bacteriophage" and "phage" are interchangeably used herein and refer to viruses which infect bacteria. Phage cannot replicate on their own.

However, upon infection of a bacterial cell, the phage can direct the cell machinery to produce more viral material. Each phage has either DNA or RNA as its genetic material, but not both.

As used herein the term "infection" and "infective" refer to the process in which a bacteriophage attaches to, and enters into a host bacteria. Infection can follow either a lytic path in which the bacteriophage propagates within the host leading to host cell lysis, or a lysogenic path in which integration of the bacteriophage genome into the host genome occurs (prophage) with no initial bacteriophage propagation and cell lysis. Preferably the term infection is used herein to refer to a lytic infection.

Taking into account these definitions, the present invention is directed to compositions and methods of using modified phage to detect bacteria. Virtually all bacteria have infecting phages that are extremely species specific.

Bacteria/Infecting Phage Combinations

Bacteria/infecting phage combinations are well known in the art and readily available in the literature. Exemplary, but in no way limiting, examples of bacteria/infecting phage combinations are described at the ncbi.nlm.nih.; evergreen.edu and phage.atcc.org web sites.

In particular, the bacteria *Escherichia coli* is infected by the following phage: *E. coli* phage T1, *E. coli* phage T4, and *E. coli* phage T5 among others as disclosed in the following exemplary references: Pecota D.C. and Wood T.K.. Exclusion of T4 phage by the hok/sok killer locus from plasmid R1. J. Bacteriol. 178: 2044-2050, 1996 PubMed: 96178971; Pecota D.C. and Wood T.K.. Exclusion of T4 phage by the hok/sok killer locus from plasmid R1. J. Bacteriol. 178: 2044-2050, 1996 PubMed: 96178971 RF44654: Schmid I. et al,. Biosafety guidelines for sorting of unfixed cells. Cytometry 28: 99-117, 1997 PubMed: 97325246; and F44807: Danziger R.E. and

Paranchych W., Stages in phage R17 infection. 3. Energy requirements for the F-pili mediated eclipse of viral infectivity. Virology 40: 554-564, 1970 PubMed: 70157093

The bacteria *Salmonella typhimurium* is infected by the following phage: *Podoviridae* that infects *Salmonella* species; Bacteriophage P22 which is also known as Phage P22 and P22 Phage as disclosed in the following references: L.S. Baron J. Gen. Microbiol. 9: 410-433, 1953 and Zinder N.D. and Lederberg J., J. Bacteriol. 64: 679-699, 1952.

The *bacteria Campylobacter jejuni* is infected by the following phage: *Campylobacter jejuni* phage 2 [J1 328]; *Campylobacter jejuni* phage 4 [J2101]; and *Campylobacter jejuni* phage 7 [L2 106] among others as described in Grajewski B.A. et al. Development of a bacteriophage typing system for *Campylobacter jejuni* and *Campylobacter coli.*, J. Clin. Microbiol. 22: 13-18, 1985 PubMed: 85261888.

The bacteria *Listeria monocytogenes* is infected by the following phage: phage of the family *Siphoviridae*; Genus "lambda-like phages"; *Listeria* phage H387 (H387); Listeria phage 2389 (2389); Listeria phage 2671 (2671); and Listeria phage 2685 (2685) phage of the Family Myoviridae Genus "T4 -like phages)" and Listeria phage 4211 (4211) as described at the ncbi.nlm.nih.gov web site.

In addition to these bacteria/phage combinations, other bacteria/infecting phage combinations are well known in the art and readily available in the literature.

Cloning in Phage

Procedures for DNA cloning in phage are well known in the art. For example, the protocol-online.net; molbio/phage/phage web and urmc.rochester.edu web sites provide detailed protocols for phage cloning. Cloning techniques utilizing M13 and lambda phage are readily known and available.

In producing the recombinant phage of the invention, a modified phage genome is prepared. First the head protein or another critical packaging protein

(gene) is mutated so it is no longer functional thereby preventing contamination problems that might occur in a lab if the phage were allowed to freely complete their life cycles. Phage can be multiplied by use of a helper phage.

Second, the phage is genetically modified so it always enters the lytic stage. In this format, the phage will not have the option of integrating into the host genome and therefore will not replicate.

Third, the phage genome is modified to contain a recombinant, randomly repeated signature or identifying oligonucleotide sequence of, for example, 30-100 bp in length. If this sequence also includes the amplification primers discussed below then it needs to be longer, up to 200 bp. It may be present in 5 to 100 or more copies depending on the repeat length and the packaging capability of the phage. This oligonucleotide sequence will be of non-phage and non-microbe origin and will act as a detection target for the recombinant phage and its host.

Generally, the signature or identifying oligonucleotide sequence will be DNA that does not share homologous sequences with the phage or associated pathogen(s) or host.

For lambda phage and other double-stranded DNA phages, DNA is isolated by procedures well known in the art such as phenol-choloroform extraction, affinity chromotography, electrophoresis, etc. Once the lambda DNA has been isolated, the DNA is digested with a restriction enzyme at an area that flanks the non-vital lysogenic genes in order to remove the genes. By removing these genes, the phage will go lytic and it also creates cloning space for an insert. Lambda DNA ends are commercially available for cloning (Packagene – Promega, MaxPlax – Epicentre, or Ready-To-Go Lambda Packaging – Amersham). Additionally some cloning vectors such as cosmids are commonly used for cloning and replication in both *E. coli* and phage.

Once the phage DNA has been digested with restriction enzymes at the site of interest and the lysogenic genes have been removed, the DNA ends are dephosphorylated. This can be accomplished using either gel electrophorsis to separate the bands coupled with gel purification. Alternatively, the enyzme digested mixture can be purified directly (following a phenol/chloroform extraction, ethanol precipitation, to inactivate the phosphatase). After phenol chloroform extraction, the phage genomic DNA is resuspended in buffer, for example 20 μl of TE.

An identifying or signature oligonucleotide is then selected for cloning. The oligonucleotide may be a synthetic oligonucleotide of a random repeat sequence or isolated DNA containing the repeat sequence. The repeat sequence is generally added to the DNA at a ratio of 1 molar insert : 1 molar phage DNA and/or 3:1 plus ligase and buffer. The solution is allowed to ligate for sufficient time to permit the ligation to take place, generally over night.

Next, the recombinant phage DNA is added to phage proteins and the phage molecule will self package forming new bacteriophages. These phage are then used to infect host cells on a plaque assay. This is done by growing up host cells (*E. coli*) in a liquid broth overnight, mixing them with the ligation mixture (recombinant phage) in solution of top agar. The mixture is poured on a nutrient agar plate and incubated overnight. The following morning plaques should be visible. Each plaque represents an independent ligation event and a functional phage. Plaques will be picked and analyzed. PCR may be utilized by targeting the inserted oligonucleotide sequence to confirm that the phage are indeed recombinant. Positive phage are grown up in mass and used as detectors of viable host cells.

The above non-limiting example is with lambda, a very common phage, with commercially available phage cloning and packaging systems. If the phage of choice has no commercially available cloning systems there are numerous ways available to one skilled in the art to construct the phage based bacterial detection

system. Two non-limiting examples are provided. First, Loessner et al., (Loessner M.J., Rees C.E., Stewart G.S., and Scherer S., 1996 Appl Environ Microbiol 62:113-1140) describes a method of introducing the *lux* gene into the *Listeria* phage A511 by using homologous recombination. A plasmid containing the *lux* gene flanked by phage homologous sequences and antibiotic resistance genes was cloned and transformed into the host *L. monocytogenes*. Phage A511 was allowed to infect the transformed *Listeria* and via homologous recombination with the plasmid, recombinant phage was created. Culture extracts were used to infect *Listeria* lawns and recombinant phage were then selected by selecting luminescent plaques. In a second non-limiting example, Waddell and Poppe (2000) (Waddell T.E. and C. Poppe, 2000, FEMS Microbiol Lett 182:285-289) constructed a plasmid that contained a transposon that was used to deliver the *luxAB* gene into ΦV10 for the detection of *Escherichia coli* O157:H7. The plasmid was used to transform *E. coli* O157:H7 (R508) that was then infected with phage ΦV10. Cells were treated with IPTG to induce the transposon to jump into the phage genome. Cells were then treated with MTC to induce the prophage to go lytic. The culture broth was filtered and samples were used to infect *E. coli* O157:H7 that were plated on selective media. Colonies that were chloramphenocol resistant, bioluminate and resisted wildtype ΦV10 infection were selected for the correct recombinate phage. This method of phage mutagesis is very effective since little was known about the phage ΦV10 genome.

Phage Insert

The nature of the insert to be cloned into the phage depends on whether the recombinant phage or products produced by it is to be used for direct detection or detection via post-phage replication amplification. If the phage is to be utilized for direct detection then a single insertion is required. This scheme utilizes phage

replication as the means that the signature nucleic acid is amplified and produces copies of the product to be detected without the need for further amplification. If the phage is to be utilized for detection via amplification then a second sequence may be inserted following the above scheme at a different location generally about 80 – 200 base pairs away. For detection via gene amplification two additional sequences are needed. These amplification primer sequences generally flank the signature sequence.

With any detection method both the signature sequence and the intervening sequence may be inserted in a single cloning step as a large insert containing all of the relevant sequences.

An inserted sequence for direct detection generally consists of 2 parts. The first portion is used for detection either directly or as a nucleic acid or protein product produced from it. The detected nucleic acid product may be DNA or RNA that has the same sequence as the insert, it may be DNA or RNA that is modified post-infection. This is the signature or identifying sequence. It may also contain filler DNA (random DNA sequence) that is used to keep the spacing between the different types of sequences optimal. The second portion is used as a spacer for the detection sequences. The two parts are generally arranged in a 12121212121212 pattern where (1) represents the first sequence and (2) represents the second spacer sequence. Sequences that find use in the invention include but are not limited to detection sequence 5' AGA ATG ACC GTA ATC CGC AGA ACC (SEQ ID NO: 1).

A complete inserted sequence for detection by post phage replication amplification generally consists of 3 parts: The first part is primer 1. The second part is a spacing sequence. This may be the same as the signature or identifying sequence. It may also (or only) contain filler DNA that is used to keep the spacing between the different types of sequences optimal. The third part is primer 2. They

are generally arranged in a 123123123123 or 123321123321 pattern with (1) being primer 1, (2) being the signature and spacer and (3) being primer 2. Sequences that find use in the invention include but are not limited to Primer 1;
5'CGAAATCGGTAGACGCTACG (SEQ ID NO: 2) and Primer 2;
5'GGGGATAGAGGGACTTGAAC (SEQ ID NO: 3), or Primer 1;
5'AGAGGCTATTCGGCTATGAC (SEQ ID NO: 4) and primer 2;
5'GTGACAACGTCGAGCACAG (SEQ ID NO: 5) or primer 1;
5'CCACAGTCGATGAATCCAG (SEQ ID NO: 6) and primer 2;
5'AGGATGATCTGGACGAAGAG (SEQ ID NO: 7).

In this case the signature sequence depends on the method used for detection. If the detection of the amplification products is done by gel electrophoresis then the primers would be the signature sequence. If detection of the amplification products is done by a direct method, such as a flourescent probe hybridization then the signature sequence could be either the primers, the intervening sequence that was specific to the probes or both.

In either case the spacer can be a random sequence. The spacer may have the probe hybridization sequence included in it. Spacer sequence alone is the same type of sequence as is used in the first method where there is only direct detection and no amplification. Examples of spacer sequences include:
ACATAAATAAAATACCATCCCAAAAAGAATGAGGAACTTTGAACCACTAGAAACA
CTAACTACCATAACAAAGCCGAAAATCTTGCGATACCATGCTATGCTGTTAAAAG
AACACAATACTCTTGACAAGAGTATATGAGAGTAGAGTATATATATAGTATATCN
GATGTTTACACAAGTGCTTGGTTATCCATAAAGTTAGTAACTATTATCTATAGTAA
TCAC (SEQ ID NO: 8) or
CACGAGCTAGTGCCATCCAACGGCCTGTTCTGCATGACCATCATGTCCAGCAA
CACGATGACCCCGTGCGCGGACGGCAAGTCTTGCGGACCGTGAACCGCGCGA

TCGGGCAGCCGCACCATGTGCTGCTCAGGGATCCGCATCGGTCGTTCGGCAAT
ATGATCCTCCAAAATCCGGCGTTTTGTCCATTGATGCAGTCTCC (SEQ ID NO: 9)

Different primers and detection sequences are used for different microorganisms. The use of different primers greatly reduces the risk of contamination or false positives. However, the spacer sequence may be the same for all. In addition the spacer may contain a specific signature sequence to identify the source of the recombinant phage.

Oligonucleotide sequences may be modified in length and sequence by procedures well known in the art in order to optimize the detection of bacteria.

Once the parameters are optimized for an inserted sequence that gives the best detection, one of ordinary skill in the art may follow routine procedures to design other sequences that have these parameters. Such sequences may be designed with computer programs that are available to one of ordinarily skill in the art. Non-limiting examples of such computer programs include GeneRunner, Primer 3 and MacVector.

Sample Analysis

The compositions of the invention can be utilized to detect the presence of bacteria in a wide variety of samples, solid, liquid and gaseous, including food, agricultural products, environmental samples and various clinical specimens. If the sample is liquid, it can be per se subjected to the procedure of the invention, or first diluted, or concentrated by centrifugation. On the other hand, if the sample is solid, it should be first liquefied (for example in water) using standard known techniques, such as by use of a blender or stomacher. The liquid or liquefied sample may, if desired, be filtered through a course paper, glass or other matrix filter to remove particulates. If the sample to be tested is an environmental sample, then swabs or scrapings of the tested surface or material are mixed in a collection buffer and then treated as a liquefied food sample. Alternatively solid samples may be added

directly. Gaseous samples would be collected by standard methods and the collection matrix added.

Kits

The modified phage of the invention may be supplied in the form of a kit. In the kit format the sample is added to the phage. Prepackaged vials containing various phage may also be supplied as a kit. Generally, all the vials are placed together so that the user cannot displace them. The sample is added to all vials at the same time. If a positive reaction occurs in vial 2, for example, the user knows that the sample contains viable microorganisms specific to phage 2. This could be repeated with multiple samples.

However, other kits would also have utility in detecting a single organism where one phage is added to a single or multiple samples.

Detection of Phage

In the methods of the invention, the recombinant phage are detected by procedures well known in the art. An increase in the number of recombinant phage is indicative of the presence of the targeted bacteria in the sample. Detection may occur by PCR techniques, hybridization techniques, electrophoreses techniques, fluorescent detection techniques, visible detection techniques, peptide nucleic acid probes, electrical detection techniques, enzyme assay techniques and others. Protein products that may be detected include but are not limited to: laccase, glucuronidase, galactosidase, polyphenol oxidase.

As with any detection procedures, a sample is first collected, and incubated in a culture media with the phage. This incubation period will generally be 1 hour with aeration at 37°C. However, it could be shorter and not require aeration. Depending on the number of target microbes in the sample, amplification could be several 1000

fold in a short period of time. It is known that most phage can replicate in 20 minutes and each phage usually generates 100 copies. Compounded with the inserted repeat, the identifying or signature oligonucleotide sequence or its protein could be amplified several thousand fold under these conditions.

After the incubation, a sample of the culture mixture is taken and may be analyzed by PCR using primers specific for the inserted primer sequences. Detection would be via a gel or by use of molecular fluorescent or non-fluorescent probes or other methods.

Alternatively, after incubation probes may be added directly to a sample of the culture mixture. By heating the media to 95°C for several minutes and allowing the sample to cool the probe will hybridize with the target and if enough target copies are present it will be detectable by fluorescence. Other types of probes will be activated during the phage replication cycles and do not require heating. The probe or detection substrate may be included in the culture media with the modified phage and do not require later addition.

The use of phage has some real advantages over standard PCR identification of microorganisms. Phage will only replicate in a viable cell. Thus the techniques of the invention can be used to access bacterial cell viability. Many phage replicate at 37 °C thus no thermal cycler maybe needed. With detection of products produced only after infection one does not have to worry about background detection. Phage can be very host specific. Thus one does not have to worry about cross reactivity and false bands. In addition, phage are very stable and an assay using them is inexpensive and easy to use.

We claim:

1. A method of detecting or identifying a viable bacterial phage host in a sample, comprising:

a) combining a modified phage with said sample to form a mixture wherein said modified phage includes one or more detectable nucleotide sequences;

b) incubating said mixture under suitable conditions to permit said modified phage to infect and amplify in said host and form amplified phage and

c) detecting said nucleotide sequence in said amplified phage to thereby detect or identify a viable host for said phage in said sample.

2. The method of claim 1 wherein said modified phage contains two detectable nucleotide sequences.

3. The method of claim 1 wherein said nucleotide sequence is RNA.

4. The method of claim 1 wherein said nucleotide sequence is DNA.

5. The method of claim 1 wherein said sample is selected from the group consisting blood, tissue, urine, saliva, food products, and contact lens solutions.

6. The method of claim 1 wherein said bacterial host is selected from the group consisting of *Listeria*, *Campylobacter*, *Escherichia coli*, *Salmonella*, *Clostridium*, *Shigella*, *Stapylococcus*, *Vibrio*, *Yersinia*, *Plesiomonas*, *Bacillus*, *Aeromonas*, *Methicillin-resistant S. aureus (MRSA)*, *Staph epidermidis*, *Salmonella typhi*, *Salmonella typhimurium*, *Shigella dysenteriae*, *Listeria monocytogenes*, *Corynebacterium diphtheriae*, *Chlamydia*, *Streptococcus pneumoniae*,

Streptococcus pyogenes, Strep agalactiae, Group A Streptococcus (GAS), Bacillus anthracis, Mycobacterium tuberculosis, M. avium, M. leprae, M. bovis, Rickettsia rickettsii, Ehrlichia chaffeensis, Borrelia burgdorferi, Yersinia pestis, Yersinia enterocolitica, Treponema pallidum, T. carateum, Trichomonas vaginalis, Neisseria gonorrhoeae, Neisseria meningitides, Enterococcus faecalis, Enterococcus faecium,, Klebsiella pneumoniae, Serratia marcescens, Enterobacter cloacae, Enterobacter aerogenes, Haemophilus influenzae, Haemophilus parainfluenzae, Legionella pneumophila, Legionella dumoffii, Pseudomonas aeruginosa, Clostridium tetani, Clostridium botulinum, Clostridium difficile, Clostridium perfringens, Bateroides fragilis, Mycoplasma pneumoniae, Mycoplasma hominis, Chlamydiae pneumoniae, Chlamydiae trachomatis, Chlamydiae psittaci, Helicobacter pylor, Campylobacter jejuni, Campylobacter coli, Bordetella pertussis, Borrelia burgdorferi, and Enterococci.

7. The method of claim 1 wherein said phage is selected from the group consisting of: *E coli* phage T1, *E coli* phage T4, and *E coli* phage T5; *Podoviridae*; Bacteriophage P22 *Campylobacter jejuni* phage 2; *Campylobacter jejuni* phage 4 *Campylobacter jejuni* phage 7 and *Siphoviridae.*

8. The method of claim 1 wherein said nucleotide sequence is detected by an amplification reaction.

9. The method of claim 8 wherein said amplification reaction is selected from the group consisting of PCR, LCR and NASBA.

10. A method of detecting or identifying a viable bacterial phage host in a sample; comprising:

a) preparing a modified phage wherein said modified phage includes one or more detectable nucleotide sequences;

b) combining said modified phage with a sample to form a mixture;

c) incubating said mixture under suitable conditions to permit said modified phage to infect and amplify in said bacterial host for said modified phage to form amplified modified phage and

d) detecting said nucleotide sequence to thereby detect or identify a viable host for said phage.

11. The method of claim 10 wherein said modified phage contains two detectable nucleotide sequences.

12. The method of claim 10 wherein said nucleotide sequence is RNA.

13. The method of claim 10 wherein said nucleotide sequence is DNA.

14. The method of claim 10 wherein said sample is selected from the group consisting blood, tissue, urine, saliva, food products, and contact lens solutions.

15. The method of claim 10 wherein said bacterial host is selected from the group consisting of *Listeria*, *Campylobacter*, *Escherichia coli*, *Salmonella*, *Clostridium*, *Shigella, Stapylococcus*, *Vibrio*, *Yersinia*, *Plesiomonas*, *Bacillus, Aeromonas, Methicillin-resistant S. aureus (MRSA), Staph epidermidis, Salmonella typhi, Salmonella typhimurium, Shigella dysenteriae, Listeria monocytogenes, Corynebacterium diphtheriae, Chlamydia, Streptococcus pneumoniae, Streptococcus pyogenes, Strep agalactiae, Group A Streptococcus (GAS), Bacillus anthracis, Mycobacterium tuberculosis, M. avium, M. leprae, M. bovis, Rickettsia*

rickettsii, Ehrlichia chaffeensis, Borrelia burgdorferi, Yersinia pestis, Yersinia enterocolitica, Treponema pallidum, T. carateum, Trichomonas vaginalis, Neisseria gonorrhoeae, Neisseria meningitides, Enterococcus faecalis, Enterococcus faecium,, Klebsiella pneumoniae, Serratia marcescens, Enterobacter cloacae, Enterobacter aerogenes, Haemophilus influenzae, Haemophilus parainfluenzae, Legionella pneumophila, Legionella dumoffii, Pseudomonas aeruginosa, Clostridium tetani, Clostridium botulinum, Clostridium difficile, Clostridium perfringens, Bateroides fragilis, Mycoplasma pneumoniae, Mycoplasma hominis, Chlamydiae pneumoniae, Chlamydiae trachomatis, Chlamydiae psittaci, Helicobacter pylor, Campylobacter jejuni, Campylobacter coli, Bordetella pertussis, Borrelia burgdorferi, and indicator groups of bacteria such as Enterococci. The invention may also be used to detect sub-species of bacteria such as *E. coli O157:H7.*

16. The method of claim 10 wherein said phage is selected from the group consisting of *E coli* phage T1; *E coli* phage T4; *E coli* phage T5; *Podoviridae*; Bacteriophage P22; *Campylobacter jejuni* phage 2; *Campylobacter jejuni* phage 4; *Campylobacter jejuni* phage 7 and *Siphoviridae.*

17. The method of claim 10 wherein said nucleotide sequence is detected by an amplification reaction.

18. The method of claim 17 wherein said amplification reaction is selected from the group consisting of PCR, LCR and NASBA.

19. A kit for bacterial detection comprising a modified phage wherein the modified phage includes one or more detectable nucleotide sequences.

20. The kit of claim 10 further including components for detecting said nucleotide sequence.

SEQUENCE LISTING

<110> Investigen DNA Biotechnologies, Inc.
Koshinsky, Heather
Zwick, Michael S.
Loessner, Martin J.

<120> COMPOSITIONS AND METHODS FOR BACTERIA DETECTION

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