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REVIEW

Bacteriophage reporter technology for sensing and detecting microbial targets

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Abstract Bacteriophages (phages) are bacterial viruses evolutionarily tuned to very specifically recognize, infect, and propagate within only a unique pool of host cells. Knowledge of these phage host ranges permits one to devise diagnostic tests based on phage–host recognition profiles. For decades, fundamental phage typing assays have been used to identify bacterial pathogens on the basis of the ability of phages to kill, or lyse, the unique species, strain, or serovar to which they are naturally targeted. Over time, and with a better understanding of phage–host kinetics and the realization that there exists a phage specific for nearly any bacterial pathogen of clinical, foodborne, or waterborne consequence, a variety of improved, rapid, sensitive, and easy-to-use phage-mediated detection assays have been developed. These assays exploit every stage of the phage recognition and infection cycle to yield a wide variety of pathogen monitoring, detection, and enumeration formats that are steadily advancing toward new biosensor integrations and advanced sensing technologies.

Keywords Bacteriophage · Bioreporter · Biosensor · Pathogen · Phage · Reporter gene

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Introduction

The detection of bacterial pathogens in food, clinical, and environmental samples typically relies on conventional culture-dependent techniques in which the microbe must physically grow and be identified on a selective and/or differential medium. Despite being slow and labor-intensive, these fundamental microbiological assays remain the benchmark of most pathogen detection schemes. However, with significant demand to detect pathogens in minutes rather than hours or days, alternative assay methods are continuously being developed, tested, and optimized for enhanced detection efficiency. Those of greatest application are the polymerase chain reaction (PCR) and the enzyme-linked immunosorbent assay (ELISA). PCR techniques rely on the detection of a target organism's nucleic acid, whereas the ELISA is an immunoassay that uses target-specific antibodies to identify organisms of interest. In both methods, the goal is to capture target pathogens either through their specific DNA signatures using a PCR probe or through antibody binding to antigens specific to the desired bacterial target. In much the same fashion, the natural ability of a phage to adsorb to, infect, and propagate within only a unique set of host cells offers another mode of capture that can be exploited to detect, monitor, and/or enumerate pathogenic bacteria. Although phage-based detection techniques have not approached the popularity of PCR and ELISA, they are making headway owing to several key benefits. Phages, unlike PCR and ELISA, are able to differentiate between living and dead cells in assays where host infectivity is exploited, they can be produced in extremely high quantities at minimal cost, and they are

sufficiently robust and stable to maintain long shelf lives. (PCR methods incorporating chemicals such as propidium monoazide have been designed to differentiate DNA derived from living versus dead cells, and reverse-transcription PCR, utilizing the less environmentally stable messenger RNA, can likewise do so, but costs, complexities, and other technical issues have made neither a routine diagnostic tool). Also, with the distinction of likely being the most numerous biological entity on the planet, there theoretically exists a phage for any bacterial target one wishes to identify. Phage-mediated detection schemes have taken on many forms, including the visualization of tagged phages as they attach to their specific bacterial host targets, using phages as delivery vehicles to transport measurable markers into target host cells, or relying on the end product amplification and measurement of progeny phages released from target cells (Table 1). Each method has proven applicable in bacterial detection schemes, but as with all assays, unique sets of advantages and disadvantages are inherent (Table 2). These methods and their state-of-the-art integrations into biosensors as new tools in pathogen diagnostics will be discussed in this review.

Detection by phage typing

The classic means of identifying bacteria via the phage to which they are susceptible is referred to as phage typing. Phages have a host range that represents the types of bacterial cells they can infect. Host ranges can be diverse, infecting across bacterial strains, species, and genera, or highly specific, infecting only within a single bacterial serotype. This broad or restricted range of bacterial host recognition is dependent on bacterial surface receptors that the phage uniquely identifies, and, depending on the phage, can consist of surface structures such as pili and flagella, surface polysaccharides, or a diverse range of surface or membrane-attached proteins. Understanding a phage's host range permits one to identify bacteria on the basis of which phage they are infected or not infected by. In a typical phage typing assay, the bacterial culture is spread on a solid growth medium and then overlaid with small drops of several different phage solutions. If the bacterium is susceptible to the phage, the bacteria will lyse owing to phage infection and therefore not grow, resulting in the formation of a cleared area called a plaque (Fig. 1). The pattern of plaque formation denotes the susceptibility of the bacterium to each phage, and ultimately allows the bacterium to be epidemiologically identified. Phage typing schemes are widely available for nearly all pathogenic microorganisms, and include diagnostic phage sets for virtually all National Institute of Allergy and Infectious Diseases (NIAID) category A, B, and C bacterial pathogens (i.e., diarrheogenic *Escherichia coli*, *Yersinia enterocolitica*, *Y. pestis*, *Bacillus anthracis*, *Campylobacter jejuni*, *Listeria monocytogenes*, *Staphylococcus*, *Clostridium botulinum*, *Clostridium perfringens*, *Vibrio cholerae*, *Salmonella*, *Mycobacterium tuberculosis*, *Brucella* sp., *Burkholderia* sp., and *Shigella* sp.).

Detection via labeling of phage DNA

Prior to infection, phages first recognize and bind to their bacterial host. This initial binding can be used as a tag to identify bacteria provided that the phage can itself be appropriately labeled. The simplest means to do so is via labeling of the phage's DNA with various commercially available fluorescent dyes. Goodridge et al. [1] demonstrated this technique by fluorescently labeling the DNA of phage LG1 with the dye YOYO-1 and using it as a biological probe to detect *E. coli* O157:H7. When these phages attached to their *E. coli* O157:H7 host, a fluorescent halo could be visualized around the *E. coli* cell. When paired with anti-*E. coli* immunomagnetic separation to isolate and concentrate the *E. coli* cells in the sample, detection limits in artificially contaminated ground beef could be obtained at approximately two colony-forming units (CFU) per gram after a 6-h preenrichment and at 10 CFU mL^{-1} in raw milk after a 10-h preenrichment. Kenzaka et al. [2] developed a similar assay using phage T4 labeled with 4′,6-diamidino-2-phenylindole (DAPI) to fluorescently detect and enumerate *E. coli* in fecally contaminated canal waters in Thailand, with side-by-side comparison with nucleotide-probe-based fluorescent in situ hybridization (FISH) detection techniques. With FISH detecting higher numbers of *E. coli* in the canal water samples than the fluorescent phage, it was theorized that only the healthy, most physiologically active *E. coli* were being tagged by the fluorescent phage. However, phages can nonspecifically adsorb to, but not infect, cells outside their host range, and can additionally adsorb to dead cells as long as the structural integrity of the cell wall is reasonably adequate. Thus, the potential for false-positive signaling would need to be considered when performing assays in this manner.

Lee et al. [3] labeled phages ΦMP1 and ΦMP2 specific for *Microlunatus phosphovorus*, a bacterium found in activated sludge, with the fluorescent dye SYBR Green and demonstrated their effectiveness at rapidly quantifying these cells directly within the activated sludge matrix (Fig. 2). Mosier-Boss et al. [4] labeled phage P22 with the fluorescent dye SYBR Gold for the identification of *Salmonella enterica* subsp. *enterica* serovar Typhimurium

Table 1 The current collection of phage-based detection assays and their key operating parameters

Method	Phage	Bacterial target	Detection limit	Response time	Test matrix	Reference
Labeling of phage DNA (YOYO-1 fluorescent dye)	LG1	*Escherichia coli* O157:H7	2 CFU g^{-1}	6 h	Ground beef	[1]
Labeling of phage DNA (YOYO-1 fluorescent dye)	LG1	*E. coli* O157:H7	10 CFU mL^{-1}	10 h	Raw milk	[1]
Labeling of phage DNA (DAPI fluorescent dye)	T4	*E. coli*	NR	30 min	Fecal contaminated water	[2]
Labeling of phage DNA (SYBR Green fluorescent dye)	ΦMP1	*Microlunatus phosphovorus*	~10^2 cells mL^{-1}	25 min	Activated sludge	[3]
Labeling of phage DNA (SYBR Green fluorescent dye)	ΦMP2	*M. phosphovorus*	~10^2 cells mL^{-1}	25 min	Activated sludge	[3]
Labeling of phage DNA (SYBR Gold fluorescent dye)	P22	*Salmonella* Typhimurium	NR	>10 min	Culture	[4]
Use of reporter gene (*luxAB*)	λ Charon 30	*E. coli*	10 cells mL^{-1}	1.5 h	Artificially contaminated milk	[7]
Use of reporter gene (*luxAB*)	λ Charon 30	Enteric bacteria	10 cells cm^{-2} or 10 cells g^{-1}	5 h	Swine carcasses and slaughterhouse surfaces	[8]
Use of reporter gene (*luxAB*)	ΦV10	*E. coli* O157:H7	NR	1 h	Culture	[9]
Use of reporter genes (*luxI* and *luxR*)	λ	*E. coli*	1 CFU mL^{-1}	10.3 h	Iceberg lettuce	[13]
Use of reporter genes (*luxI* and *luxR*)	PP01	*E. coli* O157:H7	1 CFU mL^{-1}	22 h	Apple juice	[10]
Use of reporter genes (*luxI* and *luxR*)	PP01	*E. coli* O157:H7	1 CFU mL^{-1}	12.5 h	Tap water	[10]
Use of reporter genes (*luxI* and *luxR*)	PP01	*E. coli* O157:H7	1 CFU mL^{-1}	6 h	Spinach rinsate	[10]
Use of reporter genes (*luxAB)*	P22	*Salmonella enterica*	10 CFU mL^{-1}	6 h	Culture	[14]
Use of reporter genes (*luxAB)*	P22	*Salmonella* Enteritidis	63 CFU mL^{-1}	16–24 h	Artificially inoculated intact egg	[14]
Use of reporter genes (*luxAB)*	P22	*Salmonella* Typhimurium	10^6 CFU mL^{-1}	16 h	Poultry feed, feces, litter	[15]
Use of reporter genes (*luxAB)*	Felix-01	*Salmonella*	NR	NR	Culture	[16]
Use of reporter genes (*luxAB)*	A511	*Listeria monocytogenes*	0.1 CFU g^{-1}	20 h	Chocolate pudding, ricotta cheese	[17]
Use of reporter genes (*luxAB)*	A511	*L. monocytogenes*	1 CFU g^{-1}	20 h	Shrimp, milk, cottage cheese, cabbage, lettuce	[17]
Use of reporter genes (*luxAB)*	A511	*L. monocytogenes*	10 CFU g^{-1}	20 h	Liverwurst, soft cheese	[17]
Use of reporter genes (*luxAB)*	A511	*L. monocytogenes*	1–10 CFU g^{-1}	44 h	Hard cheese, ground meat	[17]
Use of reporter genes (*luxAB)*	ΦA1122	*Yersinia pestis*	820 cells	1 h	Culture	[19]
Use of reporter genes (*luxAB)*	Wß	*Bacillus anthracis*	10^3 CFU mL^{-1}	1 h	Culture	[18]
Use of reporter genes (FLuc)	phAE142	*Mycobacterium*	10^3 CFU mL^{-1}	1–2 weeks	Sputum	[20]
Use of reporter genes (GFP)	λ	*E. coli*	~10^2 CFU mL^{-1}	4–6 h	*E. coli*/*M. smegmatis* mixed culture	[26]
Use of reporter genes (GFP)	$T4e^{-}$	*E. coli*	NR	1 h	*E. coli*/*P. aeruginosa* mixed culture	[27]
Use of reporter genes (GFP)	$T4e^{-}$	*E. coli*	NR	1 h	Sewage	[28]
Use of reporter genes (GFP)	IP008e-/2xGFP	*E. coli*	NR	6 h	Sewage	[29]
Use of reporter genes (GFP)	IP052e-/2xGFP	*E. coli*	NR	6 h	Sewage	[29]
Use of reporter genes (GFP)	PP01	*E. coli* O157:H7	NR	10 min	*E. coli* O157:H7/*E. coli* K12 mixed culture	[30]
Use of reporter genes (GFP)	$PP01e^{-}$	*E. coli* O157:H7	NR	1.6 h	Culture	[31]
Use of reporter genes (GFP)	TM4	*Mycobacterium*	<10^2 cells	4 h	Culture	[32]
Use of reporter genes (*lacZ)*	T4	*E. coli*	10 CFU mL^{-1}	8 h	Pure culture	[33]
Use of reporter genes (*inaW)*	P22	*Salmonella*	10 cells mL^{-1}	2 h	Milk and eggs	[34]
Phage amplification	Felix-01	*Salmonella*	600 cells mL^{-1}	4 h	Pomegranate rind	[36]
Phage amplification	NCIMB 10116	*Pseudomonas aeruginosa*	40 cells mL^{-1}	4 h	Pomegranate rind	[36]
Phage amplification	NCIMB 10884	*P. aeruginosa*	40 cells mL^{-1}	4 h	Pomegranate rind	[36]
Phage amplification	D29	*Mycobacterium avium* subsp. *paratuberculosis*	≤10 CFU mL^{-1}	48 h	Dairy	[37]
Phage amplification	NR	*Staphylococcus aureus*	10 CFU mL^{-1}	5 h	Blood	[80]
Phage amplification	SJ2	*Salmonella* Enteritidis	<10^4 CFU mL^{-1}	4–5 h	Pure culture	[39]

Table 1 (continued)

Method	Phage	Bacterial target	Detection limit	Response time	Test matrix	Reference
Phage amplification	SJ2	*Salmonella*	3 CFU 25 g^{-1} or 3 CFU 25 mL^{-1}	20 h	Milk powder, chicken rinses, ground beef	[40]
Phage amplification	LG1	*E. coli* O157:H7	2 CFU 25 g^{-1}	23 h	Ground beef	[40]
Phage amplification (stained with SYT09 dye)	NCIMB 10116	*P. aeruginosa*	10 CFU mL^{-1}	4 h	Culture	[41]
Phage amplification (temperature sensitive mutations)	AR1	*E. coli* O157:H7	1 cell mL^{-1}	3.5 h	Culture	[42]
Phage amplification (mutant)	Felix-01	*Salmonella*	≤10 cells mL^{-1}	3–5 h	Culture	[42]
Phage amplification	ΦA1122	*Y. pestis*	10^3 CFU mL^{-1}	4 h	Culture	[43]
Phage amplification	MS2	*E. coli*	10^4 cells mL^{-1}	2 h	Culture	[44]
Phage amplification	MS2	*E. coli*	NR	3 h	*Salmonella*/*E. coli* mixed culture	[45]
Phage amplification	MPSS-1	*Salmonella*	NR	3 h	*Salmonella*/*E. coli* mixed culture	[45]
Quantum dots	T7	*E. coli*	10 cells mL^{-1}	1 h	Culture	[47]
Quantum dots	λ	*E. coli*	1 cell	30 min	Culture	[48]
Phage-mediated cell lysis	NCIMB 10359	*E. coli*	10^4 cells mL^{-1}	1 h	Culture	[49]
Phage-mediated cell lysis	Newport	*Salmonella*	10^4 cells mL^{-1}	2 h	Culture	[49]
Phage-mediated cell lysis	AT20	*E. coli*	10^3 CFU mL^{-1}	2 h	Culture	[50]
Phage-mediated cell lysis	SJ2	*Salmonella*	10^3 CFU mL^{-1}	2 h	Culture	[50]
Phage-mediated cell lysis	Environmental isolate	*E. coli* O157:H7	10^2 cells mL^{-1}	1 h	Culture	[51]
Phage-mediated cell lysis	λ	*E. coli*	1 CFU 100 mL^{-1}	6–8 h	Culture	[53]
Phage-mediated cell lysis	B1-7064	*Bacillus cereus*	10 CFU mL^{-1}	8 h	Culture	[54]
Phage-mediated cell lysis	D29	*Mycobacterium smegmatis*	10 cells mL^{-1}	8 h	Culture	[54]
Phage-mediated cell lysis	TG1	*E. coli*	1 CFU mL^{-1}	3 h	Culture	[55]
Phage-mediated cell lysis	λ S105	*E. coli*	10^7 CFU mL^{-1}	10 min	Culture	[57, 81]
Change in conductance	AR1	*E. coli* O157:H7	$<10^6$ CFU mL^{-1}	24 h	Culture	[58]
Phage components (cell wall binding domains)	CBD-118	*L. monocytogenes*	1 CFU mL^{-1}	6 h	Turkey breast and ground meat	[60]
Phage components (cell wall binding domains)	CBD-118	*L. monocytogenes*	10 CFU mL^{-1}	6 h	Salmon, cheese, iceberg lettuce, milk	[60]
Phage components (cell wall binding domains)	CBD-500	*L. monocytogenes*	1 CFU mL^{-1}	6 h	Soft cheese	[60]
Phage components (cell wall binding domains)	CBD-500	*L. monocytogenes*	10 CFU mL^{-1}	6 h	Iceberg lettuce, cheese, salmon, milk	[60]
Phage components (cell wall binding domains)	CBD-500	*L. monocytogenes*	10^2 CFU mL^{-1}	6 h	Turkey breast, ground meat	[60]
Phage components (tail spike proteins)	P22	*Salmonella* Typhimurium	10^3 CFU mL^{-1}	30 min	Culture	[61]
Biosensor	Sapphire	*Salmonella*	10^5 CFU mL^{-1}	2 h	Culture	[62]
Biosensor	12600	*S. aureus*	10^4 cells mL^{-1}	2 h	Culture	[63]
Biosensor	SJ2	*Salmonella* Enteritidis	4,000 CFU mL^{-1}	~40 min	Culture	[64]
Biosensor	T4	*E. coli*	NR	NR	Culture	[65]
Biosensor	E2	*Salmonella*	10^3 CFU mL^{-1}	20 min	Milk or water	[72]
Biosensor	JRB7	*B. anthracis*	10^3 CFU mL^{-1}	20 min	Water	[73]

DAPI 4′,6-diamidino-2-phenylindole, *CFU* colony-forming unit, *NR* not reported

(*Salmonella* Typhimurium). Rather than visualizing the phage particle bound to the *Salmonella* cell, they instead were able to fluorescently view the naked phage genome within the host cell, which would circumvent nonspecific false-positive signaling since the actual act of infection was being observed rather than simple phage–host attachment.

Detection using reporter phages

The end result of a productive phage infection is the transfer of the phage genome into the host cell. By judiciously inserting a user-defined gene or set of genes into the phage genome and allowing the phage to infect its

Table 2 Comparative advantages and disadvantages of phage-based detection assays

Detection method	Advantages	Disadvantages
Phage typing	Uses a native, nonengineered phage	Slow
	Exceptional specificity	Laborious
	A well-proven and well-characterized assay with a long history of success	Requires upkeep of large phage sets
	No complex instrumentation needed	Interpretation of results not always straightforward
	Inexpensive	Bacterial host strain must be culturable
Labeled phage DNA	Method to label phage DNA is simple and widely applicable	Does not necessarily differentiate living from dead cells
	Availability of different-wavelength fluorescent dyes permits detection of multiple targets	Labeled phages can attach to nonhost cells or sample particulates and misidentify
Reporter phage		
Bioluminescent bacterial luciferase (Lux) reporter phage	Detects only living targets	Requires up-front genetic engineering efforts
	Extremely low background in most sample types	Usually requires addition of an exogenous substrate
	Can be a fully autonomous, real-time, continuous assay if complete *lux* operon is used	Oxygen-dependent
		Turbid sample matrix can inhibit signaling
		Legal and regulatory issues due to recombinant nature of the phage
Bioluminescent firefly luciferase (Luc) reporter phage	Detects only living targets	Requires up-front genetic engineering efforts
	Extremely low background in most sample types	Requires addition of an exogenous substrate
		Oxygen-dependent
		Legal and regulatory issues due to recombinant nature of the phage
Fluorescent (GFP) reporter phage	Detects only living targets	Requires up-front genetic engineering efforts
	Availability of different wavelength fluorescent proteins permits detection of multiple targets	Sample autofluorescence can reduce sensitivity
		Turbid sample matrix can inhibit signaling
		Legal and regulatory issues due to recombinant nature of the phage
Colorimetric (LacZ) reporter phage	Detects only living targets	Requires up-front genetic engineering efforts
	Availability of different-wavelength substrates permits detection of multiple targets	Not as sensitive as other reporter phage types
Phage amplification assays	Uses a native, nonengineered phage High specificity	An ineffective virucide leads to false-positive results
	Detects only living targets	Normal flora in the sample can outcompete helper cells
	Commercially available in kit form (*FASTPlaque*)	Sample matrix, especially if of human origin, can be inhibitory
Quantum dots	Highly sensitive owing to high quantum yield	Requires up-front genetic engineering efforts
	Availability of different-wavelength quantum dots permits detection of multiple targets	Nanomaterial-related environmental risk concerns
Phage-mediated lysis of host cells	Uses a native, nonengineered phage	In most cases, requires a lytic phage
	Straightforward, cost-effective assay	Non-phage-related lysis of cells can initiate false positives
Conductance measurements	Uses a native, nonengineered phage	Culture medium or sample matrix can interfere with measurements
	Straightforward, cost-effective assay	Lacks sensitivity

GFP green fluorescent protein

host, one can dictate to the host cell a new set of genetic instructions to carry out. These genes, referred to as reporter genes, produce an easily identifiable reporter protein product that when expressed inside the host cell allows the host cell to be easily identified. Reporter genes and their counterpart reporter proteins typically yield an easy-to-measure visual signal such as bioluminescence [bacterial (*lux*) or firefly (*luc*) luciferase], fluorescence [green fluorescent protein (GFP)], or a colorimetric signal [β-galactosidase (*lacZ*)]. It is important to remember that the reporter phage will carry but not express the reporter gene; only upon a productive infection event will the

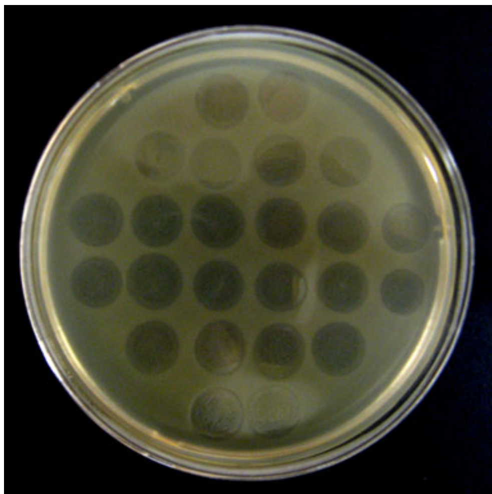

Fig. 1 Zones of clearing, or plaques, formed on a background of bacterial growth owing to productive infection of the bacteria by the phage

reporter gene be transcribed, translated, and expressed within the infected host. Also important is the test matrix one wishes to assay. As sample opacity increases, as is often the case when dealing with particulate-laden food and environmental matrices, the visual signal produced can become masked, thus affecting assay sensitivity. Optimal sensitivity, no matter what the matrix, will be dependent on the speed and efficiency of the phage in finding and infecting its host target and the sensitivity of the detector measuring the resulting signal. Since the locating of hosts by phages is a random event, which can, however, be biased by increasing the number of reporter phages in the sample, it is likely more effective to focus on advances in instrumentation to increase sensitivity. Such advances are indeed forthcoming. Conventional photomultiplier tube (PMT) and charge-coupled-device (CCD) detectors are being replaced with more sensitive intensified CCD, electron multiplying CCD, and scientific complementary metal oxide semiconductor imaging technologies (see, for example, http://www.andor.com) that acquire bioluminescent and fluorescent signals at higher fidelity with extremely low read noise. As with all innovative technology, however, expense remains a critical barrier to widespread use.

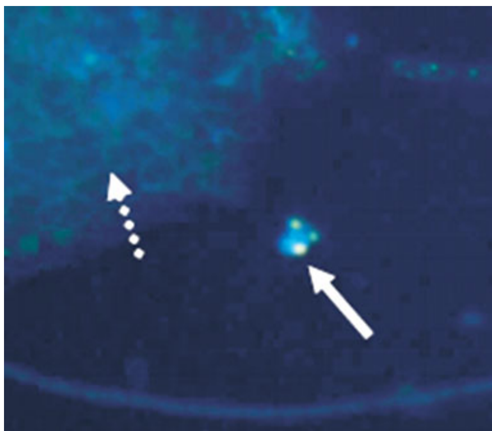

Fig. 2 SYBR Green labeled fluorescent phage ΦMP1 attached to its host *Microlunatus phosphorvorus* (*solid arrow*) against a background of an activated sludge nonhost bacterial community (*dotted arrow*). (Used with permission from Lee et al. [3])

Lux-based bioluminescent reporter phages

Bioluminescent reporter phages carrying the *lux* reporter genes cause their particular host bacteria to emit a 490-nm light signal that can be detected using any variety of PMT or CCD integrated instrument. The *lux* reporter genes are typically derived from the microbe *Vibrio fischeri* and consist of five genes listed in the order they occur in the operon, *luxC*, *luxD*, *luxA*, *luxB*, and *luxE* (or *luxCDABE*). The *luxA* and *luxB* genes (*luxAB*) produce the LuxAB protein that generates bioluminescent light from the oxidation of a long-chain fatty aldehyde in the presence of reduced riboflavin phosphate and oxygen. The remaining genes produce the LuxC, LuxD, and LuxE proteins that regenerate the aldehyde substrate required for this reaction. This aldehyde substrate can be added exogenously to the reaction, usually in the form of *n*-decanal, to trigger light production from LuxAB, thus obviating the need for inclusion of the *luxC*, *luxD*, and *luxE* genes. In phages, this is of significant advantage owing to headful packaging mechanisms that do not allow the incorporation of lengthy genes within the phage genome without detrimental effects on the phage itself. The exact length of nucleic acid that can be incorporated into a phage genome varies widely with the myriad sizes of phage genomes that nature provides, and what can and cannot be inserted is often a trial-and-error experience especially when working with genomically uncharacterized phages. However, synthetic biology has demonstrated the "cut and paste" plasticity of the phage genome and it remains to be seen how large a DNA element can be inserted when a phage genome is efficiently reorganized and reengineered [5, 6].

One of the first examples of *lux*-based reporter phage sensing was reported by Ulitzur and Kuhn [7] in 1987. They inserted the *luxAB* genes into phage λ Charon 30 for monitoring of *E. coli* and demonstrated detection down to 10 cells mL^{-1} in artificially contaminated milk within 1.5 h. Kodikara et al. [8] further tested this reporter phage and others in swab sample assays obtained from swine carcasses and slaughterhouse surfaces, where detection limits down to 10 cells cm^{-2} or 10 cells g^{-1} were achieved after 5 h, inclusive of a 4-h preenrichment. Waddell and Poppe [9] inserted the *luxAB* genes into phage ΦV10 for the specific detection of *E. coli* O157:H7 within 1 h. Taking

a different approach on *lux* reporter phage, Ripp et al. [10] designed an assay for *E. coli* detection that used two additional genes of the *lux* operon, *luxI* and *luxR*. The *luxI* and *luxR* genes are involved in the regulation of bacterial quorum sensing [11]. The LuxI protein synthesizes acyl-homoserine lactone (AHL) autoinducer molecules that freely diffuse out of cells and interact with neighboring cells, where they partner with LuxR to stimulate transcription of *luxCDABE* and *luxI*. The *luxI* gene was placed within the genome of phage λ for the general detection of *E. coli* and within the genome of phage PP01 for more specific detection of *E. coli* O157:H7 [12, 13]. Upon infection, targeted *E. coli* expressed the *luxI* gene and began synthesizing AHL molecules. Within the assay was additionally included a bioluminescent bioreporter bacterial cell that contained *luxR* and *luxCDABE*. The extracellular release of the AHL molecules by phage-infected *E. coli* cells triggered bioluminescence in the bacterial bioreporter cells, thus establishing the link between target cell presence and bioluminescent light signaling. Using the *luxI*-incorporated phage PP01, the assay detected *E. coli* O157:H7 in apple juice and tap water at 1 CFU mL^{-1} within 22 and 12.5 h, respectively. The assay was also integrated with immunomagnetic separation techniques where paramagnetic beads coated with polyclonal antibodies against *E. coli* O157:H7 (Dynal Dynabeads) were added to artificially contaminated spinach rinsate samples to assist in capture and isolation of the *E. coli* cells. In this format, and with use of a more powerful in vivo imaging CCD camera (Caliper Life Sciences IVIS), a detection limit of 1 CFU mL^{-1} was achieved in a 6-h assay.

Salmonella, being a prominent foodborne pathogen, serves as another target in *lux* reporter phage assays. Chen and Griffiths [14] designed a suite of phage P22 *luxAB* reporter phages for the detection of *S. enterica* serotypes A, B, and D_1. Detection limits in pure culture approached 10 CFU mL^{-1} after a 6-h preenrichment step. They additionally internally inoculated poultry eggs with *S. enterica* serovar Enteritidis (*Salmonella* Enteritidis) and then imaged the eggs under a BIQ Bioview image quantifier camera. Initial inoculum concentrations as low as 63 CFU mL^{-1} could be directly visualized within the egg. Thouand et al. [15] optimized the *Salmonella* assay toward a commercial kit format with proof-of-concept established in poultry feed, feces, and litter samples artificially inoculated with *Salmonella* Typhimurium, and demonstrated detection at concentrations enriched above 10^6 CFU mL^{-1} within 16 h. Kuhn et al. [16] constructed a *luxAB*-incorporated Felix-01 phage with broad host range specificity against nearly all *Salmonella*. Of critical importance in their phage reporter design was acknowledgement of the recombinant nature of the phage and its link to environmental risk assessment. They designed their reporter phage such that it would infect but not propagate within its *Salmonella* host, thus reducing the likelihood of recombinant phage progeny being dispersed at high numbers after infection events.

Another demonstrated target of phage reporters is the foodborne pathogen *L. monocytogenes*. Loessner et al. [17] designed a reporter using phage A511, which infects nearly 95% of the *L. monocytogenes* serovars responsible for human listeriosis. Cheeses, pudding, cabbage, lettuce, ground beef, liverwurst, milk, and shrimp were artificially inoculated with *L. monocytogenes* at concentrations ranging from 0.1 to 1,000 CFU g^{-1}. After a 20-h preenrichment, the A511 *luxAB* reporter phage along with the *n*-decanal substrate were added to the samples to yield detection limits as low as 0.1 cell g^{-1}. Ground beef, with its more complex microbial background flora, yielded detection limits of 100 cells g^{-1} after 20 h but this could be reduced to 10 cells g^{-1} with a longer 44-h preenrichment. A total of 348 naturally contaminated meats, poultry, dairy products, and other environmental samples were also assayed in parallel with standard plating techniques, which require much more extensive 72–96-h assay times, and *Listeria*-positive samples were found to correspond between the two methods.

Guild Associates (Charleston, SC, USA) has more recently developed diagnostic *luxAB* reporter phages for *Y. pestis* (using phage ΦA1122) and *B. anthracis* (using phage Wβ) [18, 19]. *Y. pestis* could be detected at 4,000 CFU mL^{-1} within 1 h, which in the assay corresponded to a sensitivity of approximately 800 cells. *B. anthracis* in its vegetative state was detectable at 1,000 CFU mL^{-1} within 1 h. However, the ability of *B. anthracis* to revert to a sporulated state resistant to phage infection makes it a challenge to detect. Nonetheless, when spores were revived in a germination medium, the reporter phages were able to transduce their bioluminescent signal within the first hour of incubation.

Luc-based bioluminescent reporter phages

Firefly luciferase (Luc, or sometimes referred to as FLuc or FFLuc) is a bioluminescent protein associated with the firefly *Photinus pyralis*. It catalyzes a two-step conversion of D-luciferin to oxyluciferin to generate a 560-nm bioluminescent light signal. Analogous to the addition of *n*-decanal in *luxAB* reporter assays, the D-luciferin substrate must also be added exogenously to initiate the bioluminescent response. Luc-based reporter phages have been primarily designed for the respiratory pathogen *M. tuberculosis* and determination of its susceptibility to antimycobacterial drugs, which is of paramount importance in health care to quickly determine drug resistance of a particular strain so effective treatment can be administered. Luciferase

reporter phages (LRPs) are added to *M. tuberculosis* cultures that contain or do not contain the selected antibiotic. If the antibiotic is effective and kills or limits the growth of the *M. tuberculosis* cells, then there are subsequently fewer viable host cells for the LRP to infect and, therefore, less bioluminescence being produced. LRPs in a culture with an ineffective antibiotic will have a larger population of cells to infect and thus generate higher bioluminescent outputs. Comparison of bioluminescent profiles between cultures then dictates the relative efficacy of the antibiotic. Several *Mycobacterium*-specific phages have been converted into LRPs (TM4, D29, L5, and Che12) and evaluated against conventional testing regimens. Bardarov et al. [20] assayed sputum samples with an LRP with comparison with a standard mycobacterial growth indicator tube (MGIT) assay. The MGIT assay performed better at lower *Mycobacterium* concentrations (less than 10^4 CFU mL^{-1}) but with detection occurring within a median of 9 days as opposed to 7 days with the LRP. For antibiotic susceptibility testing, results could be obtained in 3 days using LRPs, whereas 12 days was required using the MGIT assay. Banaiee et al. [21] performed a similar comparison against a BACTEC radiometric assay and showed 98% agreement between LRPs and BACTEC among 191 tested specimens. A more comprehensive comparative analysis of LRP assays can be found in the meta-analysis designed by Pai et al. [22]. Since tuberculosis is pandemic in many third world countries, Riska et al. [23] developed a minimally sustainable, low-cost Polaroid film device (the Bronx Box) that records bioluminescence emissions from LRP assays performed in multiwell microtiter plates. Using 51 clinical isolates, there was a 100% correspondence in antibiotic susceptibility profiles between the Bronx Box and standard laboratory methods, with the Bronx Box providing results in 94 h as compared with 3 weeks for the standard methods [24].

Fluorescent (GFP) reporter phages

GFP from the jellyfish *Aequorea victoria* is a popular and extensively used reporter system because it is highly stable, nontoxic, and autofluorescent, thus not requiring the addition of cofactors or substrates to initiate its 509-nm fluorescent output [25]. However, it does require activation by an excitation light source before its signal can be measured. For phage reporter applications, it is also small enough (approximately 700 base pairs) to be fully integrated into most phage genomes without affecting headful packaging limitations. The use of GFP was first demonstrated by Funatsu et al. [26] via its incorporation into the genome of phage λ for the detection of *E. coli*. Using epifluorescence microscopy, *E. coli* cells could be seen fluorescing 4–6 h after addition of the GFP λ reporter phage (Fig. 3). A similar *E. coli* detection system was designed by Tanji et al. [27] but using lysozyme-inactivated phage T4 (referred to as $T4e^{-}$). The inactivation of lysozyme activity prevented this normally lytic phage from automatically destroying the *E. coli* host cells that it was trying to detect, which ultimately increased detection limits. When GFP-incorporated $T4e^{-}$ phages were added to a culture of *E. coli* mixed with *Pseudomonas aeruginosa*, the reporter phage selectively targeted only the *E. coli* hosts, allowing them to be fluorescently visualized within 1 h against a background of nonfluorescent *P. aeruginosa* cells. In a more robust testing format, the $T4e^{-}$ reporter phages were used to directly detect *E. coli* in sewage influent [28]. Although successful, the host range of the T4 phage was not broad enough to infect all *E. coli* strains in the sewage, which highlights one of the obstacles in using phages as reporters. The host range of a reporter phage has to be restricted such that it infects only the targets one wishes to detect (i.e., no false positives) yet is broad enough to infect every target within the chosen group, species, strain, etc. (i.e., no false negatives). A single phage system likely cannot achieve these mandates, thus requiring that a suite of reporter phages be used for each bacterial target one wishes to detect. Namura et al. [29] addressed this concern by isolating two other phages from sewage and genetically incorporating into each *gfp* reporter genes. Together, these reporters demonstrated a host range covering nearly 50% of the *E. coli* sewage isolates.

Oda et al. [30] designed a GFP reporter phage for *E. coli* O157:H7 utilizing its highly specific phage PP01. Their assay discriminated between *E. coli* O157:H7 and *E. coli* K12 within 10 min on the basis of phage-mediated fluorescence. Assay sensitivity was later improved by inactivating the lytic activity of the phage [31] to provide clearer and sharper epifluorescent images. These assays additionally discriminated between healthy and stressed cells, where healthy cells emitted bright green fluorescence, whereas metabolically stressed cells emitted faded fluorescent signals. This allowed for easy, simultaneous identification of healthy cells versus cells within a viable but nonculturable state, which, when using conventional plating methods, cannot be accomplished without supplemental and time-consuming steps.

Addressing bacterial targets besides *E. coli*, Piuri et al. [32] developed a GFP-incorporated derivative of phage TM4 for the detection of *Mycobacterium* and demonstrated detection of fewer than 100 cells. They also developed a fluorescent TM4 reporter phage based on the yellow fluorescent protein ZsYellow, which emits brighter fluorescence than GFP.

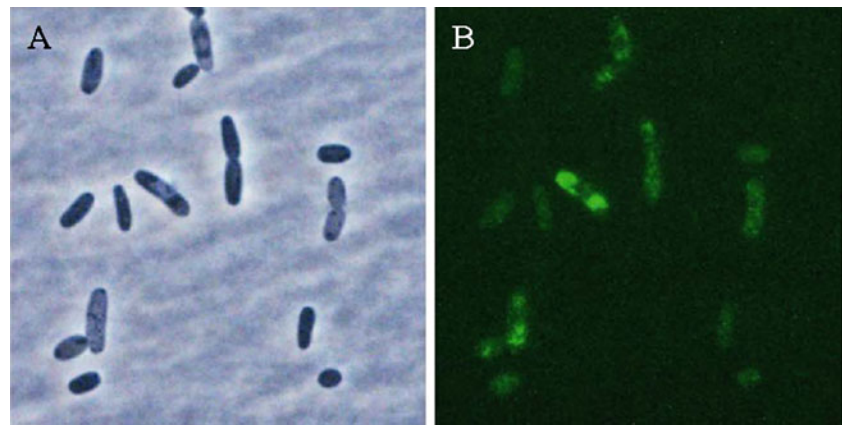

Fig. 3 **a** Optical and **b** fluorescent microscopic images of a population of *Escherichia coli* cells identified via a green fluorescent protein (GFP)-based T4 reporter phage (T4e$^-$/GFP). (Used with permission from Miyanaga et al. [28])

LacZ-based colorimetric reporter phages

The *lacZ* gene encodes a β-galactosidase enzyme that catalyzes the hydrolysis of β-galactosides. An exogenously added substrate is required that can be chosen on the basis of a user-desired colorimetric, luminescent, chemiluminescent, or fluorescent end point. Goodridge and Griffiths [33] inserted the *lacZ* gene into phage T4 for the detection of *E. coli* and demonstrated detection limits down to 100 CFU mL^{-1} in pure culture. The technique was incorporated into a most-probable-number assay that achieved detection down to 10 CFU mL^{-1} in pure culture within 8 h. The assay has also been designed around an integrated swab sampling kit, referred to as Phast Swab (Fig. 4). A surface swab or drop of liquid sample is inserted into a tube containing growth medium and incubated for up to 8 h. Immunomagnetic beads within the medium then concentrate target *E. coli* and the reporter phages are added followed 1.5 h later by a chlorophenol red-β-D-galactopyranoside substrate, which reacts with the β-galactosidase enzyme. A resulting red color indicates a positive test, whereas a yellow color indicates a negative test. With additional β-galactosidase substrates widely available, other color reactions or more sensitive luminescent, chemiluminescent, or fluorescent end points can be designed to potentially permit the detection of multiple pathogens in a single Phast Swab assay.

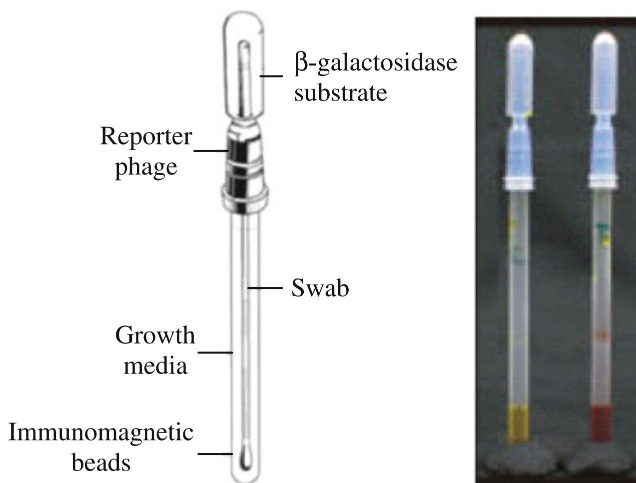

Fig. 4 The Phast Swab diagnostic kit uses a sampling swab integrated with a growth medium tube, immunomagnetic separation, and a *lacZ*-based reporter phage to detect target pathogens

Other reporter phages [ice nucleation (*inaW*)]

The *inaW* ice nucleation reporter phage cannot go without a mention because of its integration into a marketed commercial kit. The *inaW* gene encodes for ice nucleation, yielding the InaW protein, which integrates itself into the bacterium's outer cell membrane, where, at temperatures between −2 and −10 °C, it acts as a catalyst for ice crystal formation. Wolber and Green [34] inserted the *inaW* gene into *Salmonella* phage P22. Upon infection, liquid samples containing suitable *Salmonella* host cells froze when exposed to approximately −10 °C temperatures owing to the expression of *inaW*. In its commercial kit format, referred to as the bacterial ice nucleation diagnostic (BIND) assay, an indicator dye was included that turned orange if freezing occurred and fluorescent green if it did not, thus providing an easy visual end point. The BIND assay could detect as few as 10 cells mL^{-1} in artificially inoculated eggs and milk. It is, however, no longer commercially available.

Detection by phage amplification

If phages are added to a culture of susceptible bacterial hosts, infection will occur and amplified numbers of progeny phages will be released in the medium. The detection of these elevated phage numbers forms the basis for phage amplification assays. Hirsh and Martin [35] first described this method in 1983 for the detection of *Salmonella* using phage Felix-01. Elevated phage numbers

due to productive infection of *Salmonella* in the sample were detected by an identifiable peak after high-performance liquid chromatography (HPLC). Since that time, less complex end-point analyses have been adopted to bypass the costs and complexities of HPLC. For the most part, most modern phage amplification assays rely on a four-step process where the phages are first added to the sample. If appropriate bacterial hosts are present, then infection occurs. Prior to host cell lysis and release of progeny phages, a virucide is added to inactivate all extracellular free phages still in solution. The virucide is then neutralized and a population of healthy bacterial host cells (referred to as helper, sensor, or signal-amplifying cells) known to be susceptible to phage infection are added. Upon subsequent lysis of the originally infected cells, progeny phages are released and then readily infect the healthy helper cells to produce a burst of new phage synthesis. This burst of phage activity can be visualized via the formation of plaques on a growth plate. For example, Stewart et al. [36] added phage Felix-01 to a sample of *Salmonella* and incubated the mixture for 25 min to allow the phages to establish an infection within their *Salmonella* hosts. A virucide derived from pomegranate rind was then added to inactivate phages still free in solution (i.e., those that had not infected a *Salmonella* cell). After the pomegranate rind virucide had been neutralized, helper cells were added to provide healthy infectible hosts for progeny phages newly released from infected cells. As few as 600 *Salmonella* Typhimurium cells per milliliter could be detected in pure culture within 4 h on the basis of plaque counts. The assay was also performed with the *P. aeruginosa* specific phages NCIMB 10116 and NCIMB 10884, where detection of 40 cells mL^{-1} in pure culture was achieved within 4 h. The significant advantage of phage amplification assays is in their use of natural rather than genetically modified phages. No genetic engineering or other manipulations need to be performed on the phages, which equates to considerable savings in time and expense, and the often prohibitive regulatory issues inherent in using a genetically modified organism are avoided.

Phage amplification assays are most widely applied for the detection of *M. tuberculosis* using the commercially available *FASTPlaque*TB kit (Biotec Laboratories, Ipswich, UK). "Actiphage" are added to the sample for 1 h, followed by addition of the virucide for 5 min. After neutralization, a fast-growing mycobacterial cell suspension is added, referred to as the sensor cells, to act as recipients for new phage infection. Resulting infections are quantified as plaques on a top agar growth medium. Two large-scale studies verified the detection of 65–83% of confirmed *M. tuberculosis* infections in sputum samples within 2 days using this assay [22]. Foddai et al. [37] recently optimized the *FASTPlaque*TB assay for specific detection of *M. avium* subsp. *paratuberculosis* – a pathogen of significance to the dairy industry owing to its association with Johne's disease.

A *FASTPlaque*-Response kit is also available for establishing rifampicin antibiotic resistance in *M. tuberculosis*. The sample is preincubated in the presence or absence of rifampicin and then processed using the phage amplification steps described above. If the *Mycobacterium* cells are resistant to rifampicin, the number of plaques enumerated will be similar in both samples. If the cells are sensitive to rifampicin, the number of plaques in the rifampicin-treated sample will be less than the number in the rifampicin-free sample. The susceptibility of other antituberculosis drugs (isoniazid, ethambutol, streptomycin, pyrazinamide, ciprofloxacin) can also be assayed [38].

MicroPhage (Longmont, Colorado, USA) has developed a phage amplification diagnostic for *Staphylococcus aureus*. The *S. aureus* specific phage are mixed with blood or other specimen samples and phage amplification in the presence of *S. aureus* is detected via a dipstick-type immunoassay. Methicillin-resistant *S. aureus* can also be differentiated from methicillin-susceptible strains on the basis of the same principle used in the *FASTPlaque*-Response kit described above. The company is actively developing assays for the determination of other antibiotic resistance patterns in *S. aureus* as well as diagnostics for a variety of other medically important bacterial pathogens. Its products are commercially available in the European Union but not yet in the USA.

Another version of the phage amplification assay relies on immunomagnetic separation of the target cells, which bypasses the need for addition of a virucide. Use of virucides can be problematic because none are universally effective, and poor performance produces false-negative results. Favrin et al. [39] concentrated *Salmonella* Enteritidis with anti-*Salmonella* paramagnetic beads and then added phage SJ2 for 10 min. By essentially locking the *Salmonella* Enteritidis cells to the beads, they were able to magnetically hold them in solution while performing washing steps that removed free phages. Thus, no virucide was necessary. Additionally, rather than relying on the visualization of plaques, they devised a simpler optical density measurement for ascertaining infection of the helper cells. A decrease in optical density indicated that helper cell concentrations were declining owing to infection and lysing by phages, whereas an increase in optical density indicated an unaffected and growing population of helper cells. The assay was also tested in artificially contaminated skimmed milk powder, chicken rinses, and ground beef with an average detection limit of 3 CFU 25 g^{-1} or 3 CFU 25 mL^{-1} in a total assay time of 20 h, inclusive of preenrichment incubations [40]. It was also applied to the detection of *E. coli* O157:H7 using phage LG1 and anti-*E. coli* paramagnetic beads, with a detection limit of 2 CFU 25 g^{-1} in ground beef within a 23-h assay [40].

Phage amplification end points have also been linked to cell staining methods as a means of determining host cell viability. Jassim and Griffiths [41] used a fluorochromic BacLight live/dead assay where viable cells were stained green with SYTO9 dye and dead cells were stained red with propidium iodide. The propidium iodide stain penetrates only cells with damaged cell membranes, as would occur after phage infection. The dyes are added to the sample after the phage amplification assay and the resulting ratio of green to red cells signifies the magnitude of cells susceptible to phage infection. Their assay was tested with *P. aeruginosa* cells and phage NCIMB 10116. Detection in pure culture was achieved within 4 h at a detection limit of 10 CFU mL^{-1}.

Ulitzur and Ulitzur [42] developed a highly novel phage amplification assay that took advantage of phage mutant repair mechanisms to ensure that end-point plaque formation was due only to infected target bacteria. Phages possessing amber mutations (phage Felix-01 for *Salmonella*), ultraviolet-light-irradiated mutations (phage OE for *E. coli*), or temperature-sensitive mutations (phage AR1 for *E. coli* O157:H7) were constructed. These phages could not form plaques on their host cells unless their mutations were repaired by recombination or complementation, thereby bypassing the need to wash and/or centrifuge the assay samples to remove free phages. For example, two temperature-sensitive phage mutants were mixed with *E. coli* O157:H7 at the permissive temperature (37 °C) followed by incubation at their restrictive temperature (42 °C) to prevent further infection cycles. Subsequent plaque formation was therefore only possible if the mutation had been repaired since any remaining mutant phages, owing to their temperature sensitivity, could not form plaques at 42 °C. The number of plaques thus reflected the number of *E. coli* host cells in the sample. Detection was achieved down to 1 cell mL^{-1} in a 3.5-h assay. Similar co-infection strategies with the other phage mutants yielded detection limits of ten or fewer target cells per milliliter in 3–5-h assay formats.

Several other adaptations of the phage amplification assay have been reported. A variation based on quantitative real-time PCR (qPCR) end-point measurements was recently described for the detection of *Y. pestis* [43]. Phage ΦA1122 was added to samples, whereupon productive infections yielded phage bursts. The increase in phage numbers was detected via qPCR primers designed against the phage genome. Detection of 1,000 CFU mL^{-1} was established within 4 h. Madonna et al. [44] used matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) to identify the molecular weight signature of the phage capsid protein. *E. coli* in pure culture was concentrated by immunomagnetic separation and then infected with phage MS2. Analysis of 1 μL of sample by MALDI-TOFMS was sufficient to detect the MS2 capsid protein, providing a detection limit of approximately 10^4 *E. coli* cells per milliliter in an assay time of 2 h. Rees and Vorhees [45] additionally demonstrated simultaneous MALDI-TOFMS detection of *E. coli* and *Salmonella* using respective protein signatures on their MS2 and MPSS-1 phages. Guan et al. [46] combined phage amplification with a competitive ELISA to detect *Salmonella* Typhimurium using phage BP1 and a biotinylated version of BP1. *Salmonella* cultures were incubated with wild-type BP1 phage and resulting phage supernatants were added to ELISA microtiter plates coated with *Salmonella* Typhimurium smooth lipopolysaccharide, to which the phage attached. The biotinylated version of the phage was additionally added, and could be detected by the colorimetric substrate 3,3′,5,5′-tetramethylbenzidine peroxidase. If excess wild-type BP1 phages were present, owing to the availability of suitable *Salmonella* host cells, then few biotinylated phages would attach and a weak colorimetric signal would be detected. If no target *Salmonella* were present, then BP1 replication would not occur and excess biotinylated phages would bind to the smooth lipopolysaccharide to yield an intense yellow color.

Detection using quantum dots

Quantum dots are fluorescent probes consisting of colloidal semiconductor nanocrystals. Their high quantum yield and excellent photostability have made them popular as probes and labels in biological and molecular imaging. Their integration into phage reporter applications, however, has been surprisingly slow. In 2006, Edgar et al. [47] first reported on the use of nanoengineered phage-conjugated quantum dots to detect *E. coli*. Phage T7 was modified to express a biotinylation peptide on its major capsid protein, allowing T7 phage progeny produced after *E. coli* infection to become biotinylated in vivo. Once released from their *E. coli* host, biotinylated progeny phages were captured via streptavidin-functionalized quantum dots that could be visualized by fluorescence microscopy or flow cytometry. Owing to the high quantum yield, a single-quantum-dot-conjugated phage could be microscopically observed. Detection limits using flow cytometry approached 10 cells mL^{-1} within an assay time of 1 h. With the availability of quantum dots of differing wavelength emissions, multiplexed imaging of several different target pathogens within a single sample, using phages of differing specificity, would be practical. Yim et al. [48] recently validated and modeled quantum dot–phage complexes using a biotinylated λ phage and its host *E. coli*.

Detection via phage-mediated lysis of host cells

The lysis of host cells after phage infection releases not only a multitude of new progeny phages but also the

intracellular contents of the cell, whose detection and measurement can be used as an indicator of phage infection events. Thus, by adding phages with known host ranges to a sample and monitoring the presence or absence of intracellular host cell constituents, one can determine if infection occurred and, therefore, identify host cells in the sample. An example of this method is the phage-mediated adenylate kinase assay. The adenylate kinase enzyme reversibly controls the conversion of ADP to ATP and AMP (2ADP ⇋ ATP + AMP) and is present in virtually all cells. Blasco et al. [49] created a phage-based assay whose end point relied on adenylate kinase driven conversion of ADP to ATP. The *E. coli* specific phage NCIMB 10359 or the *Salmonella* specific phage Newport were added to bacterial cultures, and, if suitable host cells were present, infection, lysis, and release of adenylate kinase occurred. ADP was then added to drive the adenylate kinase-mediated reaction toward the generation of ATP, and resulting ATP pools were detected using a commercially available firefly luciferase assay. Detection limits approached 10^4 cells mL^{-1} of *E. coli* or *Salmonella* within an assay time of 2 h or less. Wu et al. [50] later optimized assay incubation times and phage concentrations to increase the detection limits to 1,000 CFU mL^{-1}. Squirrell et al. [51] combined the adenylate kinase assay with immunomagnetic separation to achieve detection limits of 100 cells mL^{-1}. Luna et al. [52] used the method essentially in reverse to detect phages rather than host cells. Somatic coliphages are a group of phages used as indicator organisms to signify fecal contamination in water. By adding an appropriate host (*E. coli* WG5) for these phages to water samples and monitoring for host cell lysis via the accumulation of adenylate kinase, they could establish the presence of coliphages.

Neufeld et al. [53] used electrochemistry to measure amperometric changes in solution due to phage-mediated cell lysis. Infection of *E. coli* by a lytic version of phage λ ultimately led to the release of cellular components, such as the enzyme ß-D-galactosidase. ß-D-Galactosidase can be measured amperometrically with a potentiostat via the addition of the substrate *p*-aminophenyl-ß-D-galactopyranoside to yield the product *p*-aminophenol, which is oxidized at the carbon anode. *E. coli* could be detected within 6–8 h at a detection limit of 1 CFU 100 mL^{-1}. Yemini et al. [54] used the same principle to detect *Bacillus cereus*, where lysis by phage B1-7064 caused cellular release of the enzyme α-glucosidase, as well as *Mycobacterium smegmatis* using phage D29 and the cellular release of ß-glucosidase. Theoretically and advantageously, any phage–host combination can be detected using this method as long as the phage is lytic and an appropriate electrochemically detectable enzymatic marker is released by the target cell. However, a single phage would likely be insufficient to infect across the spectrum of target bacterial cells desired and false-negative signals arising from cross-infections or naturally lysing cells would have to be accounted for. Neufeld et al. [55] addressed some of these concerns in their assays using a phage-encoded alkaline phosphatase enzyme that had to be delivered to the host cell to be expressed. Thus, only after an active infection event would the enzyme be synthesized and then later released by the cell during lysis. This assay could detect a single *E. coli* CFU per milliliter in less than 3 h in both pure and mixed cultures.

Relying on the release of intracellular constituents necessarily requires waiting for the phage to find, adsorb to, infect, propagate within, and finally lyse its host cell. This ultimately adds time to the duration of an assay. In the sensing of phage-triggered ion cascade (SEPTIC) assay, electrochemistry is used to measure microscopic voltage fluctuations occurring after a phage has injected its nucleic acid into its host cell. Thus, the initial establishment of phage infection is detected rather than its end result. Immediately after injection, the bacterial host will emit approximately 10^8 ions into the surrounding medium, and this release can be measured electrochemically using two thin metal film microelectrodes. Dobozi-King et al. [56] demonstrated the assay using *E. coli* as the target cell in a 5-µL nanowell sensor chip [57]. Although in these experiments *E. coli* was detected at 10^7 CFU mL^{-1}, a theoretical detection limit of 1 CFU mL^{-1} was hypothesized on the basis of potential improvements in fluid conductivity and/or reductions in thermal noise. The assay significantly benefits from the use of a wild-type phage, so no costly or time-consuming genetic manipulations are required, and either lytic or lysogenic phages can be used since host cell lysis is not necessary.

Detection via changes in conductance

Chang et al. [58], on the basis of the knowledge that growth in a microbial culture can be monitored electrochemically by measuring changes in electrical parameters occurring as complex growth medium substrates are broken down into smaller highly charged molecules such as acids, hypothesized that the presence of phages within a bacterial culture, provided that suitable host cells were present, would impede culture growth and therefore directly affect growth medium composition. Thus, by comparing conductance measurements between phage-supplemented and phage-free samples or between phage-specific and non-phage-specific bacterial cultures, one could easily screen samples for the presence of phage-specific pathogens. Phage AR1 and its *E. coli* O157:H7 host were used to demonstrate the technique. Pure cultures of *E. coli* O157:

H7 or non-O157:H7 cells at 10^6 CFU mL^{-1} with or without phage AR1 addition were placed in test tubes fitted with platinum electrodes and conductance measurements were taken every 6 min. The resulting conductance curves could discriminate between *E. coli* O157:H7 and non-O157:H7 cultures within a 24-h period.

Detection using phage components

Rather than using the complete, viable phage, specific recognition proteins of the phage can be isolated and applied in detection schemes. Phage-encoded endolysins, for example, are enzymes that destroy the host cell wall and promote the release of the newly replicated phage virions. Endolysins contain a cell wall binding domain (CBD) that enables the enzyme to recognize and bind to specific cell wall structures, and this recognition can be of a sufficiently narrow spectrum to allow endolysin CBD moieties to be used for the specific detection of defined bacterial targets. Schmelcher et al. [59] fused variously colored fluorescent proteins to endolysin CBDs specific among serovars of *L. monocytogenes*. Fluorescently tagged CBDs were added to mixed serovar *Listeria* cultures, where they bound to and tagged their targeted cells to yield a multiplexed image delineating each serovar by color (Fig. 5). Detection directly from food sources (cheese, milk) was also demonstrated using CBD fluorescent tagging in combination with a CBD magnetic separation technique previously developed by Kretzer et al. [60]. Paramagnetic beads coated with a broad-spectrum CBD were first used to capture and isolate *Listeria* populations from the sample [faster (48 h versus 96 h) and with better efficiency than conventional antibody-coated magnetic beads], followed by fluorescent imaging with the narrow-spectrum CBD reporters to rapidly (approximately 15 min) distinguish which serovars were present in the sample. CBD-coated magnetic beads can also be used in combination with more typical quantitative end points such as PCR with excellent detection limits (1,000 CFU mL^{-1} or lower). Proof-of-concept application of endolysin CBDs for bacterial targets besides *Listeria*, such as *Bacillus* and *Clostridium*, have also been reported [60]. Endolysin CBD detection assays are, however, limited to Gram-positive bacterial targets since endolysins derived from phage-infecting Gram-negative bacteria do not exhibit analogous specificity owing to the presence of an outer membrane that blocks access to the cell wall.

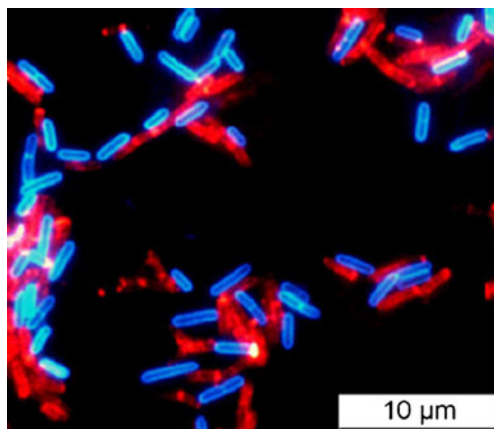

Fig. 5 Addition of *Listeria*-specific phage-derived cell wall binding domain proteins tagged with differently colored fluorescent reporters permits multiplexed discrimination within mixed-serovar *Listeria* cultures. (Used with permission from Schmelcher et al. [59])

Phage tail fiber components have also been used to capture and identify bacterial targets. The commercially available VIDAS® UP assay from BioMerieux/Hyglos uses a recombinant phage tail protein reagent to capture and detect *E. coli* O157. More recently, Singh et al. [61] engineered recombinant tail spike proteins from phage P22 and demonstrated their efficiency in specifically binding to and capturing *Salmonella* Typhimurium cells at rates sixfold better than those obtained by intact wild-type P22 phage.

Phage-based biosensors and immobilization strategies

A biosensor is an integrated device that incorporates a biological component with a transducer element to detect, monitor, and communicate the presence of a chemical, physical, or biological target. It generally consists of three main components: the biorecognition element, which recognizes and binds the target of interest with high selectivity, the transducer, which converts the binding reaction into a measurable signal, and the output system, which amplifies and displays the signal in a useful form. The self-contained architecture of the biosensor permits it to be used in an online and/or portable fashion, and ideally with features such as rapidity, accuracy, simplicity, and low cost. A number of biosensors have been designed for the detection of microbial targets, typically incorporating antibodies or nucleic acids as the biorecognition element and piezoelectric, electrical, surface plasmon resonance (SPR), or optical waveguides as the transducer. The selectivity of phages has been exploited for biosensor applications but to a lesser extent primarily owing to the lack of effective immobilization methods for adhering the phage to the transducer. However, progress is being made and phage-integrated biosensor applications are evolving. An early version of phage immobilization used phage Sapphire to capture *Salmonella* [62]. Suspensions of phage were passively immobilized onto a polystyrene strip and used as a dipstick device to capture *Salmonella* Typhimurium in solution. In a more biosensor-relevant format,

Balasubramanian et al. [63] physically adsorbed phage 12600 onto the gold surface of an SPR sensor (the Spreeta sensor marketed by Texas Instruments, which uses SPR to detect changes in refractive index due to receptor–ligand binding interactions) for the detection of *S. aureus*. *S. aureus* cells were pumped across the Spreeta channel, where they contacted and attached to the immobilized phage. Non-phage-specific bacterial cells, in this case *Salmonella* Typhimurium, simply passed through the device and were deposited as waste. Detection of 10^4 *S. aureus* cells per milliliter could be achieved in near real time. Such detection limits are rather poor, primarily owing to the phage not being properly oriented. For a tailed phage to capture its host, its tail, which serves as the recognition receptor for the cell, must face outward, whereas its head should ideally be immobilized inward toward the solid phase. This was accomplished by Sun et al. [64] by biotinylating the phage coat proteins and using the high affinity of biotin for streptavidin to constrain the phage in a more-or-less oriented fashion. They biotinylated phage SJ2 and coated it onto streptavidin-labeled magnetic beads for use as a biosorbent to capture *Salmonella* Enteritidis. Gervais et al. [65] similarly biotinylated phage T4 and oriented it on a gold surface on an electric cell–substrate impedance (ECIS) chip interface for the detection of *E. coli* (Fig. 6). As more *E. coli* cells attached to the ECIS chip surface, the flow of ion current was further restricted, resulting in an increase in measured impedance. Tolba et al. [66] developed an analogous phage binding approach using head protein linked cellulose binding domains as anchor points. Singh et al. [61] immobilized their recombinant tail spike proteins from phage P22 on a gold substrate and, in combination with flow-through SPR, were able to detect *Salmonella* Typhimurium in pure culture down to 1,000 CFU mL^{-1}.

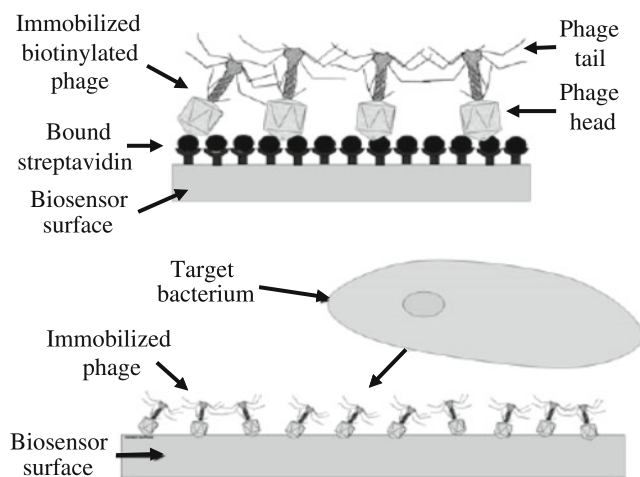

Fig. 6 Oriented deposition of phages on a biosensor surface using biotin–streptavidin chemistry allows the phage tail fibers to remain free to bind to receptors on the host cell surface. (Adapted from Gervais et al. [65])

Alternatively, phages in their natural state can be used and physically oriented by taking advantage of the phage's charge, which in most cases is net negative owing to the negative charge of the head. Electrostatic interaction can therefore be used to align the phage on appropriately modified surfaces, as demonstrated by Cademartiri et al. [67] with their electrostatically driven physisorption of phage T7 on silica supports. Natural phage proteins can also be used to directionally orient an unmodified phage, for example, on glutaraldehyde-activated surfaces [68]. Antibodies can also be designed against phage coat proteins to assist in proper alignment, as was demonstrated by Solis et al. [69] in their development of patterned lithographic microarrays of antibody–M13 phage complexes.

With effective phage immobilization methods only recently maturing, the parallel development of phage-based biosensors has yet to find its true stride, and only a handful have been developed. This includes the SPR Spreeta biosensor [63] and the ECIS chip [65] described above, the use of phage λ to detect *E. coli* on the basis of amperometric detection of a reporter enzyme [53], the use of phage T4 to detect *E. coli* on screen-printed carbon electrode microarrays, and the use of phage Newport to detect *Salmonella* on conducting polymer electrodes [70]. However, additional biosensor formats have been developed using phages derived from phage display libraries as the biorecognition elements. Phage display represents a powerful technique for creating libraries of phages each of which displays on its surface a different recognition peptide for a different biological target, thus functioning much like an antibody in its interaction with an antigen [71]. Thread-shaped filamentous phages such as M13, f1, and fd are engineered with user-specified nucleic acid spliced within their coat protein genes. Subsequent expression of phage genes and assembly of phage components yields mature phage particles that display foreign peptides on their surface. These "landscape" phage libraries can express a countless number of antigen recognition sites that act as probes for pathogenic targets, as well as resting endospores, toxins, chemicals, and viral agents. Lakshmanan et al. [72] immobilized a phage library clone selective for *Salmonella* Typhimurium on a magnetoelastic sensor surface. Binding of free *Salmonella* to the immobilized phage resulted in a change in mass that could be measured by a consequent shift in resonance frequency. In a flow-through format using artificially inoculated water or milk, detection limits of approximately 1,000 CFU mL^{-1} could be obtained within 20 min. A similar magnetoelastic sensor for the detection of *B. anthracis* endospores in water yielded identical detection limits [73].

Commercial marketability and the criticisms of phage-based assays

Relatively few phage-based detection assays are commercially available. This lack of commercialization is somewhat surprising considering the diversity of experimental phage diagnostics that demonstrate valid proof-of-concept and cost-effectiveness that often meets or exceeds the metrics of currently used culture-dependent or molecular methods. In addition, there is an extensive parallel recognized application of phages focused on biocontrol (exploiting the lytic nature of phages or phage products to eradicate bacteria of consequence to food safety and environmental sanitation [74]) and phage therapy (using phages or phage products to control bacterial infections relevant to human and veterinary medical therapies [75]), which further promotes phage-based biotechnologies. The reluctance to use phages as biodetection agents can be attributed to several key perceived or factual disadvantages. Primary among these is the concern over host resistance to phage infection. A subpopulation of phage-resistant mutants within a sample can lead to false-negative results. As well, the emergence over time of phage-resistant bacterial populations can render a phage-based detection assay obsolete, as has already occurred with our arsenal of antibiotics. Both concerns are valid; however, nearly all culture-dependent and culture-independent testing regimens can be criticized for generating false-negatives: growth of atypical colonies on selective and differential media can be incorrectly identified as false negatives, sample matrices can inhibit PCR amplification, mutations in PCR primer sequence targets can occur, damaged or genetically altered antigenic epitopes on target cells can inhibit antibody recognition in immunological tests, etc. Criticisms of false-positive identifications are similarly broad in scope. All assays have their unique set of limitations and proof-of-concept studies have demonstrated that phage-based assays do not comparatively exceed the conventional boundaries of specificity and sensitivity, and therefore are probably unfairly criticized in this regard. Furthermore, the extensive population of phages from which one can choose and the ability to use multiphage cocktails to target host cells lessens the incidence of host acquired phage resistance. However, if genetically engineered phages are used, the effort and expense of creating multiphage cocktails for each pathogen of interest would be daunting, but achievable, especially as the numbers of sequenced phage genomes increases. Evolution also dictates that population-level host acquired phage resistance would not necessarily be a common occurrence. Phages have likely evolved to recognize host cell receptors that are robust and not easily lost by the cell, which in corollary are the same receptors that the host cell does not want to lose because they afford some degree of survival advantage [76]. In addition, if bacteria do successfully acquire phage resistance, phages in turn will likely evolve countermeasures to overcome these resistances [77].

No matter how efficient a phage is in recognizing and infecting its host cell, the phage must by necessity first find its host cell, and this is a random event. Under low target bacterial densities, this can occur far too slowly for a detection assay to be practicable or, worse yet, may not occur at all. For example, a rough approximation predicts that it would take 1,000 years for one phage to randomly find one bacterium in 1 mL of liquid [78]. Add in particulates from food, environmental, or diagnostic samples that would nonspecifically bind up free phages and the chances of productive phage–host encounters diminish even further. The odds of productive encounters can, however, be increased by adding excess phages to the sample, which, when considering the ease and low cost associated with producing high-titer phage stocks, is practical. However, host cells can undergo "lysis from without" when lysins produced by large numbers of absorbed phages damage the cell wall to such an extent that the cell ruptures, thereby adding to the false-negative pool. The balance between too few and too many phages would require close scrutiny.

Another concern with using phages is their considerable propensity to transfer genetic material among their host cells via the process referred to as transduction. With transduction estimated to occur at the phenomenal rate of 2×10^{16} times per second in aquatic environments, phages are quite possibly one of the primary drivers of bacterial evolution [79]. The widespread use of reporter phages carrying recombinant genes would therefore entail some degree of risk assessment. However, use of recombinant reporter phages in a laboratory kit format does not infer widespread environmental release or substantial risk, so their application within the confines of a food quality control or diagnostic laboratory does not appear overwhelming. Moreover, genetic engineering tricks can be used to limit recombinant phages from propagating, although this adds to the complexity of developing such phages, or naturally nontransducing phages can be used, if such phages exist or can be isolated for the pathogen of interest [16, 78]. The use of wild-type phages or phage components would, however, be less problematic and attests to these types of phages/phage components being used in commercially available kits from Biotec Laboratories, Microphage, and BioMerieux. Conversely, from a commercial standpoint, wild-type phages are difficult to patent.

Conclusions

With every aspect of the phage life cycle being exploited for sensing applications, the versatility afforded by phage-mediated detection methods holds promise for a wide

selection of assay formats to meet user-defined needs. This, in combination with the natural specificity of phages for their host cells and the massive number of phages from which one can choose represents a powerful set of tools for bacterial pathogen diagnostics that can operate with sufficient sensitivity and speed to complement and/or replace conventional detection methods. Indeed, detection limits of less than one bacterial cell justify their effectiveness and continued research, development, and optimization. Phage display and the ability to engineer landscape phage libraries capable of detecting not only living pathogens but toxin, spore, and viral agents as well augments the sensing repertoire. Integrations with novel biosensor prototypes, however, remains limited and will clearly need to be intensified to drive the power of phage assays toward marketable devices applicable to the biotechnological, food, medical, and agricultural industries.

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