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Bacteriophages as biocontrol agents of food pathogens

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Bacteriophages have long been recognized for their potential as biotherapeutic agents. The recent approval for the use of phages of *Listeria monocytogenes* for food safety purposes has increased the impetus of phage research to uncover phage-mediated applications with activity against other food pathogens. Areas of emerging and growing significance, such as predictive modelling and genomics, have shown their potential and impact on the development of new technologies to combat food pathogens. This review will highlight recent advances in the research of phages that target food pathogens and that promote their use in biosanitation, while it will also discuss its limitations.

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Introduction

Bacteriophages represent one of the most abundant biological entities in nature and have long been recognized for their potential use as therapeutic agents [1]. In recent years overprescription of antibiotics and the concomitant development of antibiotic-resistant 'super-bugs' have highlighted the need for alternative strategies to combat infectious diseases. Consequently, a lot of phage research in the past two decades was aimed at assessing whether phage can be used to eliminate undesirable bacteria. Traceability is a requirement in modern food production, incorporating every step in the production process, commonly known as the 'farm to fork' concept (European Commission White paper on Food Safety, January 2000. See: http://ec.europa.eu/food). Phages are omnipresent and are accidentally, yet regularly, consumed through ingestion of water and food. For this reason they are presumed to be safe as undesirable effects have not been reported. This, together with their specificity, makes them excellent tools for food safety purposes.

The 'farm to fork' concept identifies quality assurance steps at which bacterial contamination may occur, and which also represent critical points where phage treatments may be applied. The most frequently encountered food pathogens belong to one of the four dominant genera, *Salmonella*, enterotoxigenic *Escherichia coli*, *Campylobacter* and *Listeria*, along with less common infections by *Clostridium* spp., *Staphylococcus aureus*, *Streptococcus suis* and *Cronobacter sakazakii* [2–5]. Phages targeting strains of each of these species have been identified and this review will discuss the pros and cons of the use of phages as biocontrol, biosanitation and detection agents.

Isolation of phages targeting food pathogens

Pathogenic bacteria with broad-spectrum antibiotic-resistance have become a considerable public health hazard, in particular to elderly, young and immuno-compromised. Consequently, phage research is focused on phages infecting such pathogenic bacteria. In terms of food pathogens, phages against *Campylobacter jejuni*, *Salmonella enterica*, *Listeria monocytogenes* and *Streptococcus suis* have been isolated and characterized, and each of these exonerate the therapeutic use of these phages.

Campylobacter jejuni is the most commonly isolated *Campylobacter* species and is the main aetiological agent of enteric infections. Many procedures outlining the isolation of phages of *Campylobacter* have been reported, generally with limited success [6,7]. An improved isolation procedure for *Campylobacter* phages was recently described in which pre-enrichment of the phages with potential host strains is supplemented with divalent cations to promote phage adherence to the host [8]. The pre-enrichment step in this study permitted the isolation of 43 phages which were assessed against a number of *Campylobacter coli* and *C. jejuni* strains, revealing their broad host range and thereby their potential to enhance food safety.

Phages have also been isolated capable of infecting *Salmonella* strains that are associated with food-borne illnesses, including *Salmonella enterica* Typhimurium, albeit with low frequency and with narrow host ranges, which will limit their use as pre-harvest biocontrol agents [9]. In contrast, phages recognizing TolC as their receptor can prevent adherence of *Salmonella* serovars to their host [10••]. TolC is involved in the adhesion and invasion of

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host intestinal epithelial cells [11] and is produced by all serovars of *Salmonella*, therefore protection against a wide number of strains should be afforded by phages that target TolC receptors. Such ubiquitous targets may prove useful in the development of a broad-spectrum phage application. Furthermore, a phage, named IMM-001 isolated from surface water, was shown to be specific for the enterotoxigenic *E. coli* colonization factor [12••]. This phage does not infect common enteric bacteria including non-toxigenic *E. coli* indicating that its effect in natural habitats would not be of ecological concern [12••].

The successful isolation of phages of other food-borne pathogens, such as *Streptococcus suis* (associated with swine production) and *Staphylococcus aureus* (associated with dairy products) is a further proof of the resurgence of phages as biotherapeutic agents [13–16]. However, the lack of standardization of such isolation procedures may result in the selection of a subpopulation of the phages present in any given sample and may not be a true reflection of the entire phage population. The improved isolation procedure incorporating an enrichment step for the selection of *Campylobacter* phages described above highlights this problem. Furthermore, the addition of divalent cations to enrichment media should be considered as a standard component to phage isolation procedures as they have frequently been shown to be essential for phage–host interactions. In order for a phage to be selected for application in food for human consumption, several aspects should be considered. Primarily, the phage should be strictly lytic and display minimal transduction frequencies. Ideally, the full genome sequence of the phage should be known to fully assess the suitability of the phage to application in food systems. Secondly, the phage's host range should be ascertained since in certain cases, for example with coliphage IMM-001, it is beneficial that the phage specifically infects enterotoxigenic *E. coli* strains, thereby negating any negative impact on the intestinal microflora of the consumer if it were to be applied. In many other cases, a broad host range is desirable therefore this criterion needs to be assessed for each particular phage depending on the desired outcome. Additionally, if a phage is proposed to be used in a particular food matrix, its ability to function in such food model systems should be evaluated. Finally, in light of the temperature-dependent nature of phage–host interactions, it is vital that the efficacy of the phage at the temperature of intended use is determined (Fig. 1). In keeping with the above-mentioned criteria, the use of several bacteriophages or their encoded lysins has been studied to ascertain their effectiveness in food models and host challenge assays [17••–25].

Application of phage or their lysins as biocontrol agents

The Centre for Disease Control and Prevention (CDC) has reported that the leading causes of death due to food-borne bacterial pathogens are caused by *Listeria* and *Salmonella*, followed closely by *E. coli* (*E. coli* O157:H7, in particular) and *Campylobacter jejuni*, and consequently studies on the use of phages against pathogenic bacteria in food systems have mainly focused on these organisms. Phages have a wide range of potential applications as biocontrol agents in foods and as alternatives to antibiotics in animal health, where regulations for their use may not be as stringent compared to human therapeutic applications. The use of phages and their lysins as biocontrol agents for food safety applications has been recently reviewed [26]. Indeed, the U.S. Food and Drug Administration (USFDA) approval in 2006 of List-Shield™, the LMP-102 phage preparation from Intralytix for the control of *L. monocytogenes* on ready-to-eat (RTE) foods [17••], was a major breakthrough. Also the anti-listerial agent, bacteriophage Listex P100 (phage P100), has been approved by the USFDA for *L. monocytogenes* control in meat and cheese products [18•], while its anti-listerial activity of Listex P100 has also been demonstrated for raw salmon [22] and fresh channel catfish fillets [23]. A recent comprehensive evaluation of *Listeria* phage as biocontrol agents in RTE foods found that biocontrol of *L. monocytogenes* can be achieved with virulent broad-host range phages [20•]. Additionally, Listex has been approved for use in Switzerland in cheese-making and also as processing aids in keeping with European legislation on food safety [27]. Recent studies of other such phages, such as A511, demonstrate the potential for their application in foods [20•,28••]. However, it should be noted that a temperature-dependent phage-resistance phenotype was observed in broad-host range phages of this genus [29], and it may therefore be useful to determine phage efficacy at low temperatures to investigate the anti-listerial activity during food storage conditions [30] (Code of Federal Regulations, 2008). While the efficacy of specific phages of *Salmonella* and *Campylobacter* spp. at refrigerated temperatures has been investigated, the required dose of phage is relatively high and may incur regulatory difficulties. The majority of studies examine the lytic ability of the phage at the optimum growth temperature for the pathogen. However, in order to obtain information for the potential application of a phage, efficacy studies should be performed at the temperature at which the foodstuff is prepared, processed or stored. While the addition of phages as processing aids bypasses current EU legislation, increasing pressure to generate comprehensive food safety regulations may reverse the current position for such phage-based products. Human phage therapy trials are controlled by strict regulatory and administrative guidelines and are for this reason very costly, in some cases exacerbated by the incorrect classification of phages as human viruses [31]. The implications of these regulatory restrictions for phages in biosanitation of food products are vast and may inhibit their introduction into certain markets. However, permission was granted for a

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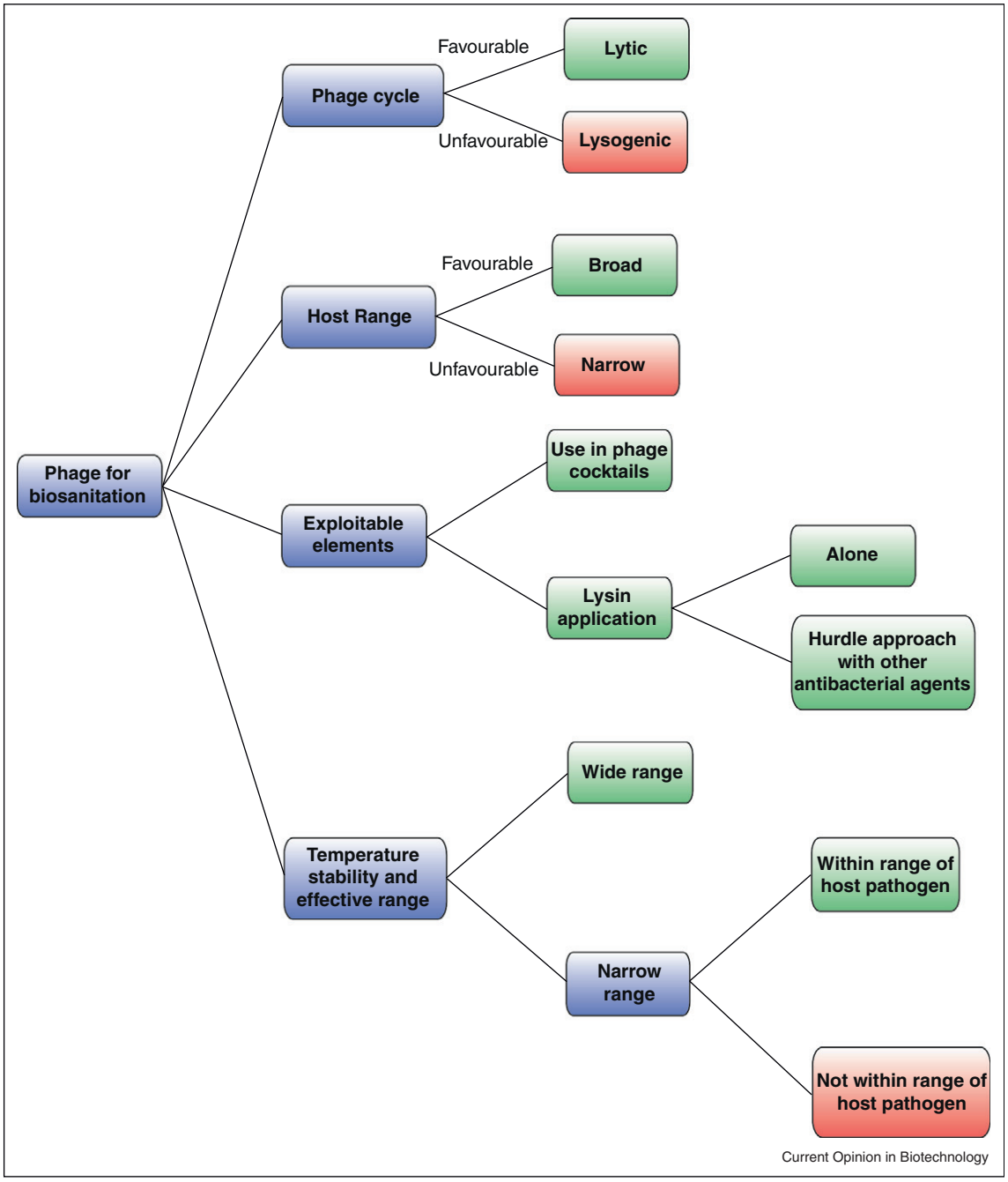
Figure 1



The schematic illustration represents a decision tree that may be applied during the selection of specific phages with potential application in pre-harvest or post-harvest phage-biosanitation treatments. In blue are points of question, while in green and red are highlighted favourable and unfavourable outcomes, respectively.

clinical trial in the UK for the treatment of ear infections by phage therapy and such studies may set the foundation for other such studies both in the areas of human phage therapy and food biosanitation [31]. Food safety legislation regarding phages is largely undefined in both Europe and Asia and studies such as those mentioned above may aid in the development of proper regulatory classification of phages that may permit their controlled use in the future.

Studies on the biocontrol of *E. coli* O157:H7 have focused on preharvest intervention strategies, i.e. administration

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of phages directly to animals, since cattle and sheep are the primary reservoirs for this pathogen [32,33]. A cocktail of O157:H7-specific phages isolated from commercial cattle faeces was shown to reduce O157:H7 populations in the cecum and rectum, but not in the rumen, of experimentally inoculated sheep [33]. In a more recent study, oral and rectal administration of a cocktail of four O157-specific phages was evaluated for the control of faecal shedding of O157:H7 by experimentally inoculated steers. In this instance, the orally treated steers produced the lowest number of *E. coli* O157:H7 culture-positive samples compared with rectal administration or a combination of oral and rectal treatment, but this number was only nominally lower than that for the control group [21]. These studies offer good prospects for the use of phages as a preharvest intervention as part of an integrated pathogen reduction scheme.

A combinatorial approach of bacteriophages and their lysins, together with natural antimicrobial substances has been employed to target emerging *Salmonella* serovars in recently published biocontrol studies. Such a hurdle approach using a combination of *Enterobacter asburiae* JX1, an organism with antagonistic activity against *Salmonella*, and a *Salmonella*-specific phage cocktail was shown to be effective against *Salmonella javiana* associated with tomatoes [24] and various *Salmonella* serovars associated with mung bean sprouts [25]. Garcia *et al.* recently demonstrated synergistic effects between the endolysin LysH5, encoded by the *S. aureus* phage phi-SauS-IPLA88, with the bacteriocin nisin to effectively inhibit *Staphylococcus aureus* in pasteurised milk [19••].

In an attempt to bypass the negative effect of gastric conditions, *Salmonella* phage Felix01 was encapsulated in chitosin–alginate microspheres and was shown to withstand simulated gastric transit by retaining its full infectivity, thus demonstrating the suitability of this delivery system for relevant applications in human therapy [34]. The identification of an *E. coli* O157:H7-specific phage with genetic and morphological similarities to Felix01 may allow the extension of such studies to other pathogens [35].

Phage biocontrol strategies in food thus appear to be a promising alternative to traditional food safety and preservation measures. However, most studies have been performed with artificially inoculated crops, animals or foods, and 'real world' conditions were not examined as a rule, which will be a prerequisite for adoption in the preharvest and post-harvest stages of food production.

Phage-based pathogen detection systems

Because of regulatory constraints on the direct application of phages as biopreservatives, a 'prevention' rather than 'cure' approach with phage biosensors may represent a realistic alternative to post-infection treatments. Several novel methods for the detection of food-borne pathogens have been described and the use of phages as biosensors is one of the fast-growing areas of research relating to food safety [36•,37–39,40•,41]. Phages and phage proteins (such as the tail-spike protein of *Salmonella* phage P22), being immobilized onto various surfaces, can act as biodetectors [36•,37,41]. Adaptation of the tail-spike proteins to improve their affinity will allow optimization of these proteins as detection tools [40•]. Furthermore, R-type pyocins, which are high molecular weight phage tail-like protein complexes produced by some *Pseudomonas aeruginosa* strains, were shown to have bactericidal effects against *E. coli* O157:H7 isolates when fused to O157-specific tail-spike proteins of an unrelated *Podoviridae* phage named V10 [39]. The specificity of action and the flexibility for further adaptation to several targets illustrate the potential of such systems in the food safety and health-care sectors.

Phage and phage protein immobilization on atomic force microscope probes coupled with single molecule force microscopy has demonstrated the strong affinity and specificity of such immobilized factors, corroborating previous studies in this area, at least those relating to *Salmonella* phages [37]. Biotin and cellulose-binding domains have been incorporated into recombinant phage T4 particles to allow attachment to various compounds such as cellulose and streptavidin magnetic beads. While these T4 derivatives were shown to have reduced infectivity, the system is more sensitive than many other pathogen-detection methods, thus warranting further research [38]. The immobilization of phages of a number of food-borne pathogens on silica particles may also allow the development of relatively inexpensive and flexible food pathogen-monitoring systems [36•]. The latter application requires no chemical or genetic alteration of the phages and the phages retain their infective capabilities while being bound to the silica surface [36•]. These phage-based biosensors may be coupled to PCR, bioluminescence or agglutination-based assays [38,39,41–43] (see Table 1). Similarly, bioluminescence and impedimetric methods have been developed to rapidly detect phage infection of particular hosts in order to determine the suitability of phages in phage cocktail preparations for biotherapeutic use [44,45]. The suitability of phages in biodetectors for field work has long been supported given their thermal and pH stabilities, and methods for the optimal preparation of purified phages without modification of the phage capsid charge have recently been described [46]. All such phage-based biodetection systems were achieved through significant developments in genomics and predictive technology, areas of emerging interest in phage biology.

Genomics and predictive technology: the way forward

Understanding genome content and stability is a prerequisite if phages are to be included in products for food

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Table 1

Summary of recent developments in phage immobilization and pathogen detection technologies

Immobilization material	Phage or phage protein as probe	Target organism	Detection/ visualisation system	Detection limit	Reference
Gold-coated surfaces	Phage P22 and P22 tail-spike protein	*Salmonella* Typhimurium	SYTO staining and fluorescence imaging	10^3 cfu/ml	[40•]
Organosilane monolayer	Phage P22 and P22 tail-spike protein	*Salmonella* Typhimurium	Atomic force microscopy	ND	[37]
Streptavidin-coated magnetic beads and cellulose	Recombinant phage T4 with head modifications	*E. coli*	Real time PCR	800 cells	[41]
Gold	Phage T4	*E. coli*	Fluorescence microscopy	ND	[57]
Nano-aluminium fiber-based filter	Wild-type and modified T4	*E. coli*	Bioluminescence	6×10^3 cfu/ml	[38]
Paramagnetic beads	Endolysin-derived protein domains	*Listeria monocytogenes*	Real time PCR and culture-based methods	10^2 to 10^3 cfu/ml	[43,57]
Silica particles	φAG2, φAG8, φAG11, φAG3	*E. coli*, *L. innocua*, *S. enteriditis*, *S. boydii*	Electron microscopy	ND	[36•]
	Phage A1122 with *lux* tag	*Yersinia pestis*	Lux system bioluminescence	10^2 cells	[42]

safety applications, since phages may encode virulence factors with the potential for phage conversion of bacterial targets. Recently, the 134 494 bp genome of the obligately lytic *Listeria* phage A511, which can significantly reduce *Listeria* numbers in liquid and on solid foods, was shown to lack genetic functions required for genome integration and transduction. The inability of A511 to transduce is probably because of its long terminal repeats that apparently prevent accidental packaging of host DNA, making A511 a suitable candidate for biocontrol applications [47]. More recently, comparative analysis of *Listeria* phage genomes revealed that they show extensive mosaicism within building blocks of conserved position [48]. Comparative genome analysis of two *Campylobacter* phages with potential to be used for biocontrol revealed evidence for a lineage of virulent bacteriophages that predate upon *Campylobacter* strains [49]. The 42 526 bp genome of the lytic staphylococcal phage phi-SauS-IPLA88, harbouring the endolysin LysH5, revealed point mutations in the lysogeny control-associated genes, explaining its strictly lytic behaviour [19].

One of the major drawbacks of phage therapy is the possible emergence of phage-resistant derivatives. Little is known about the frequency of emergence of these phage-resistant bugs and perhaps more careful consideration should be applied to this in selecting phages for potential application. However, while this is a pertinent issue in the acceptability of phages in human consumption, it has been noted in strains of *Salmonella enteriditis* that phage-resistant mutants become avirulent through the loss of the O-Polysaccharide layer, which is required for phage adsorption [50]. A similar finding has also been observed in the fish pathogen *Pseudomonas pecoglossida* [51]. However, this issue remains a concern and requires evaluation on an individual basis. To overcome the issue of developing phage-resistance through phage therapy, predictive modelling of phage–host interactions and the so-called 'mutant selection window' has been the focus of recent studies [52–54]. Such model systems provide a wealth of information relating to the level of phages required in a given system and the optimal conditions for infection. Studies on *Salmonella* Typhimurium and *Campylobacter jejuni* illustrated the importance of assessing a variety of conditions when selecting phages for their application as biotherapeutics [52,54]. Modelling also allows prediction of the emergence of phage-resistant variants within a host population [53]. Furthermore, a study on the co-evolution of phages and their hosts in *Pseudomonas fluorescens* indicates that an increased mutation rate is not directly linked to an increase in co-evolution rate, and that the kinetic relationship between phage and host should be assessed on an individual basis [55]. Such research is imperative in surmounting regulatory barriers towards full acceptance of the use of phages against food pathogens.

Conclusions and future perspectives

The increasing demand for rapid pathogen detection systems and alternatives to antibiotic treatment is driving the strong resurgence of interest in phage therapy and phage-based detection systems. Knowledge on pathogen receptors and colonization factors provides excellent targets for phage-derived treatments as demonstrated in *E. coli* and *Salmonella* systems, and may form the basis for phage treatments against a wider variety of food-borne pathogens. Phage genome analysis will allow easy identification of phages lacking integration machinery, which will facilitate safe and effective use of phages as biocontrol agents, although long-term studies on the emergence of resistant mutants and consumer safety should be performed for any phage to be applied commercially.

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Ethics statement

The authors declare that no competing interests exist.

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