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Evidence Project Final Report

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Project identification

1. Defra Project code: CTX1129
2. Project title: Pesticide Research and Development Requirements (Plant Protection Products): Strategy Development for Bacteriophage-Based Biopesticides
3. Contractor organisation(s): Advanced Pest Solutions Ltd.
4. Total Defra project costs (agreed fixed price): £ £47,942
5. Project: start date 1/2/13
   end date 31/3/14

6. It is Defra's intention to publish this form.

   Please confirm your agreement to do so..... **YES X NO** ☐

   (a) When preparing Evidence Project Final Reports contractors should bear in mind that Defra intends that they be made public. They should be written in a clear and concise manner and represent a full account of the research project which someone not closely associated with the project can follow.

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Executive Summary

7. The executive summary must not exceed 2 sides in total of A4 and should be understandable to the intelligent non-scientist. It should cover the main objectives, methods and findings of the research, together with any other significant events and options for new work.

Bacteriophage are natural enemies of bacteria. They are viruses that very specifically infect bacteria; they are not capable of infecting mammalian cells. Interest in bacteriophage as natural, non-toxic and highly effective bactericides was initiated in the early 1900's as alternatives to antibiotics and currently, there are a number of bacteriophage-based preparations used in medical- and veterinary situations to combat antibiotic-resistant infections. These characteristics also confer considerable potential to bacteriophage as biocontrol agents against bacterial pathogens of agricultural and horticultural crops. Benefits over conventional pesticides include their high level of specificity to the target (often at bacterial strain level) and low risk profile for operators, consumers and the environment. Bacteriophage are the most abundant self-replicating organisms in our environment, present in significant numbers in water sources, on food and as normal commensals of humans and animals. Humans and animals naturally ingest large numbers of bacteriophage, with no known adverse effects.

Despite the potential of bacteriophage for the control of plant pathogens, their commercial potential has not been fulfilled to date. In addition to technical issues associated with the high level of bacteriophage specificity, against a disease or disease complex that may be caused by a number of bacterial strains or even species, commercial issues exist in that there is no clear regulatory route for such applications. It is anticipated that within the EU, bacteriophage-based PPP would be considered microbial biopesticides. Bacteriophage, however, have characteristics quite different from other microorganism-based products and to allow these product to be sold as PPP in the EU, there is an urgent need to develop a relevant policy and guidance for the regulation of bacteriophage as PPP. The overall objective of this study was to review the current regulation (EC) No. 1107/2009 and microbial guidance documents for bacteriophage biopesticides and to define where adaptations are required to the standard microbial data requirements. Supporting evidence was reviewed from commercial bacteriophage products registered outside of the EU.

Regulation (EC) No 1107/2009 applies to substances, including micro-organisms having general or specific action against harmful organisms or on plants, parts of plants or plant products, referred to as active substances. The definition of micro-organism is: "any microbiological entity, including lower fungi and viruses, cellular or non-cellular, capable of replication or of transferring genetic material". Bacteriophage as viruses can reasonably be considered as micro-organisms. However, bacteriophage are not specifically referred to in Regulation (EC) No 1107/2009 nor in the implementing regulations. They have been examined by the European Food Safety Authority in relation to their use in controlling

bacterial pathogens on food of animal origin when used as processing aids but there has been no published consideration of bacteriophage used against plant pathogens.

The closest guidance document available for bacteriophage is that for baculoviruses, which has already been included in Annex I of Council Directive 91/414/EEC (SANCO/0253/2008 rev. 2). Baculoviruses are natural pathogens of insects and other arthropods. They have a very selective, narrow host range and are incapable of infecting mammalian cells. For these reasons, baculoviruses were considered a special regulatory case by the EU and the present document summarises the characteristics of bacteriophage that warrant a similar regulatory case to be made. Specifically, the extreme host specificity of bacteriophage, their ubiquitous nature in the environment, their inability to infect or cause any effects in mammalian cells and their lack of effects on non-target organisms is discussed in relation to potential data waivers/adaptations. It is anticipated that these would facilitate the construction of a logical regulatory framework, allowing the full commercial potential of bacteriophage as plant-protection products to be realised. Reference is made to successful registrations of bacteriophage-based products for the control of plant bacterial pathogens in the USA and Canada. Within these, the relevant authorities (EPA and PMRA) separately concluded that the products, when used in accordance with good agricultural practice, presented minimal/negligible risks to operators, consumers or the environment.

Project Report to Defra

8. As a guide this report should be no longer than 20 sides of A4. This report is to provide Defra with details of the outputs of the research project for internal purposes; to meet the terms of the contract; and to allow Defra to publish details of the outputs to meet Environmental Information Regulation or Freedom of Information obligations. This short report to Defra does not preclude contractors from also seeking to publish a full, formal scientific report/paper in an appropriate scientific or other journal/publication. Indeed, Defra actively encourages such publications as part of the contract terms. The report to Defra should include:
   - the objectives as set out in the contract;
   - the extent to which the objectives set out in the contract have been met;
   - details of methods used and the results obtained, including statistical analysis (if appropriate);
   - a discussion of the results and their reliability;
   - the main implications of the findings;
   - possible future work; and
   - any action resulting from the research (e.g. IP, Knowledge Exchange).

OBJECTIVES

Bacteriophage are naturally-occurring, highly specific and environmentally-acceptable bactericides, providing a non-toxic, low risk and highly effective method of managing plant-pathogenic bacteria. Benefits over conventional pesticides include their high level of specificity to the target and low risk profile for operators, consumers and the environment. They are the most abundant self-replicating organisms in our environment, present in significant numbers in water sources, on food and as normal commensals of humans and animals. Humans and animals naturally ingest large numbers of bacteriophage, with no known adverse effects. Bacteriophage have been granted GRAS status in the USA and their use approved for a number of food processing and packing industries, in addition to agricultural applications.

It is anticipated that within the EU, bacteriophage-based PPP would be considered microbial biopesticides. Bacteriophage, however, have characteristics quite different from other microorganism-based products and to allow these product to be sold as PPP in the EU, there is an urgent need to develop a relevant policy and guidance for the regulation of bacteriophage as PPP. This should take into account the safety and benefits of bacteriophage as novel PPP. Common safety concerns associated with bacteriophage are based upon a lack of understanding about potential host-range, longevity in the environment and the potential to affect human or animal health. The overall objective of this study was to identify such evidence gaps and highlight where guidance can be developed to support regulatory approaches and policy in relation to bacteriophage-based products.

The approach taken was to carry out a desk study detailing the current microbial PPP regulation and guidance, and the proposal of a regulatory strategy for bacteriophage-based biopesticides. Strategic guidance, a literature review and the preparation of a Regulatory Strategy Document was carried out by Dr Roma Gwynn (Rationale Biopesticide Strategists).

Specific evidence objectives were:

1. To establish a series of project planning and review meetings between all parties contributing to the study; all of the following objectives were dependent on having a robust project management strategy in place.
2. To assess the current regulation (EC) No. 1107/2009 and microbial guidance documents for bacteriophage biopesticides.
3. Following review of (2), to develop a literature review protocol and define key review questions to allow a targeted literature search to be carried out.
4. To carry out a systematic review of the literature identified in (3).
5. To carry out a relevant IP audit regarding the commercial development of bacteriophage relating to food production, food safety and PPP.
6. To review the commercial/regulatory state-of-the-art regarding developments of bacteriophage relating to food production, food safety and PPP.
7. To collate the project's data and information into a final report and bacteriophage biopesticide strategy document.

COMPLETION OF OBJECTIVES

All project objectives above have been met.

METHODS USED

1. **Project Planning and Review Meetings**. Regular project meetings were held between the project partners for project planning and progress development. (**Milestone 1**: meeting schedule and project kick-off meeting).
2. **Assessment of Current Regulation (EC) No. 1107/2009 and microbial guidance documents for bacteriophage biopesticides.** The current regulations and guidance associated with microbial plant protection products were discussed between the project partners and each criterion assessed to determine whether it is suitable for assessing bacteriophage-based products. Particular attention was paid to those criteria which do not 'fit' bacteriophage products and also, on the identification of data gaps (Milestone 2). The assessment findings were used to initiate a targeted literature search.
3. **Targeted Literature Search and Review**. Following on from objective (2), key words were defined to allow a search of peer-reviewed literature relevant for bacteriophage and the areas identified in (EC) 1107/2009 that appeared inappropriate and potentially warranting a new guidance. (**Milestone 3**: identification of key words, as part of a project meeting; **Milestone 4**: completion of literature search). Initially, key words were relatively narrow but these identified very few papers and hence,

they were widened to allow a greater range of titles to be identified (Box 1). Several databases were evaluated (including PubMed and Web of Science) but only CAB abstracts gave rise to sufficient papers. The final search that was used to derive the papers considered in this report was performed on 12-09-2013 with BIOSIS Previews 1985 to 2013 Week 40, CAB Abstracts 1973 to 2013 Week 35, Ovid MEDLINE(R) In-Process & Other Non-Indexed Citations and Ovid MEDLINE(R) 1946 to Present (sub-contracted to FERA). The outputs were put into Research Manager then into an Excel spreadsheet.

Box 1. Literature search key words

Initial key words:

Search No.	1st Keyword	2nd Keyword	3rd Keyword	4th Keyword
1	+ Bacteriophage	+ Ecology	+ Mode of Action	- viruses
2	+ Bacteriophage	+ taxonomy		
3	+ Bacteriophage	+ persistence	+ survival	+/- leaf
4	+ Bacteriophage	+ persistence	+ survival	+/- foliage
5	+ Bacteriophage	+ persistence	+ survival	+/- soil
6	+ Bacteriophage	+ persistence	+ survival	+/- ultra violet
7	+ Bacteriophage	+ persistence	+ survival	+/- temperature
8	+ Bacteriophage	+ persistence	+ survival	+/- water

Final key words:

Search 1:
(bacteriophage) AND (persistence OR survival OR ecology) AND (soil OR soils): **173 abstracts**

Search 2:
(bacteriophage) AND (persistence OR survival OR ecology) AND (leaf OR leaves OR foliage): **25 abstracts**

4. **Systematic Literature Review.** Based upon the conclusions made in objective 2 and the subsequent literature search (3), data which could be used to support regulatory waivers were reviewed (by Rationale), according to EFSA guidelines ("Submission of scientific peer-reviewed open literature for the approval of pesticide active substances under Regulation (EC) No 1107/2009"). (**Milestone 5**: completion of literature review). All of the abstracts were reviewed and relevant papers obtained to form the basis of the discussion and conclusions below.
5. **IP Audit.** Relevant IP (patents, trademarks) relevant to bacteriophage associated with food production, food safety and PPP were reviewed as part of the overall assessment of the current state-or-the-art of bacteriophage and to identify potential freedom-to-operate issues. A previous audit commissioned by APS was used as a background for the current update, with professional input from Marks & Clerk LLP. (**Milestone 6**: completion of IP audit and report).
6. **Review of the Commercial/Regulatory State-of-the-Art**. The current commercial developments of bacteriophage technology targeted at food production, food safety and potential PPP were reviewed, both within and outside of the EU and relevant information is included in the discussion below. (**Milestone 7**: completion and reporting on the review).
7. **Bacteriophage Biopesticide Strategy Document.** Rationale has produced a regulatory strategy document for bacteriophage (**Milestone 8**), detailing knowledge gaps, proposing data waivers and suggesting guidance for new methodology development. Information within this report has been abstracted below. The full report will be available for further discussion with appropriate regulatory bodies.

RESULTS AND DISCUSSION

Assessment of Current Regulation (EC) No. 1107/2009 and Related Microbial Guidance Documents

Within the EU, one of the most important ways of protecting plants and plant products against harmful organisms, including weeds and of improving agricultural production is the use of plant protection products (PPP). Conventional control of bacterial plant diseases has traditionally consisted of bactericides such as antibiotics and copper-based compounds. There is, however, a desire in the EU to reduce the use of antibiotics for plant protection uses and there are increasing examples of problems associated with

copper-based products, whose mode of action centres on the inhibition of bacterial enzymes though oxidative damage (Götz *et al.*, 1994). There are efficacy issues with copper-based products (including maintaining longevity on the plant surface) and resistance to the treatments is often apparent, developed through their long-term, widespread application (Pohronezny *et al*., 1994; Scheck *et al*., 1996) and therefore, requiring increased doses for effective control (Campbell *et al.*, 1997). This causes further problems, since higher concentrations being associated with plant toxicity and damage, yielding crops unmarketable (Harling & Sutton, 2002). Concerns have also been raised regarding copper accumulation and contamination of soils (Koller, 1998).

Therefore, there is an increasing demand for alternative active substances to combat plant bacterial pathogens. One potential alternative is bacteriophage; 'natural' antimicrobials which are the most ubiquitous organisms in the world and totally inert to anything other than their target bacteria, making them the perfect biocontrol agents. Key USP's include their extreme specificity, safety and biodegradability. Production and scale-up processes are also relative straightforward. Bacteriophage are naturally-occurring viruses, found abundantly in food, water, soil and among human bacterial flora. They are highly specific in their antibacterial activity (i.e. they will not infect beneficial bacteria). We safely ingest large numbers of bacteriophage in our diet (species barriers minimise the direct bacteriophage interaction with human tissues) and they have been used as therapeutic agents in humans for >80 years with no ill effects. The concept of combating plant pathogens by means of bacteriophage can be addressed at all production stages but although they have great potential in agricultural production, particularly as part of integrated disease management strategies, their commercial potential has not yet been fully realised, partly due to uncertainties associated with regulatory approval pathways. Despite this, given the potential of bacteriophage, a number of commercial enterprises are developing products with active substances based on bacteriophage and there are examples where this work is supported by both UK and wider EU research funding.

Bacteriophage (or 'phage') are viruses that specifically infect bacteria. They are obligate intracellular parasites of bacteria and reproduce by using their host's biosynthetic pathways. Their mode of reproduction is key to their potential as alternative antimicrobials. Metabolically inert until adsorption to the membrane of a host bacterium, 'lytic' bacteriophage inject their nucleic acid into the cell, 'overriding' the cell's genome to produce new bacteriophage which cause cell death as they 'burst' into the environment to continue the cycle (Box 2). On the other hand, lysogenic bacteriophage embed themselves into the genome of their bacterial host, establishing a stable relationship with the bacteria that they have infected (Walker, 2006) and hence, are of less interest for pathogen control.

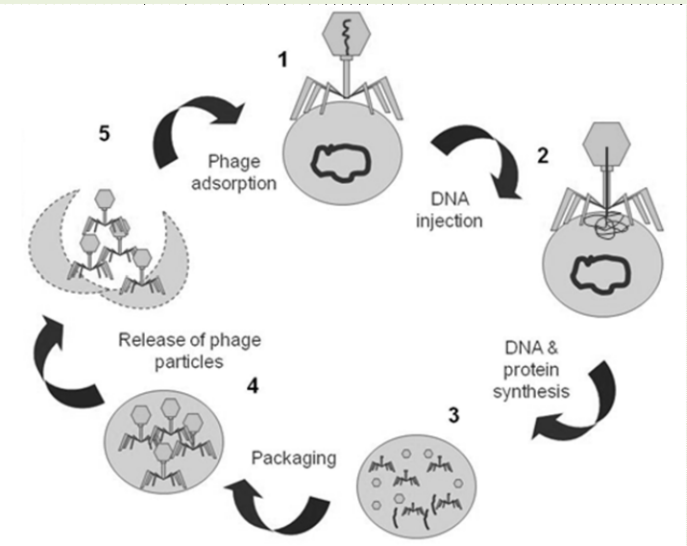
To ensure a high level of protection of both human and animal health, in addition to the environment, whilst at the same time safeguarding the competitiveness of Community agriculture, PPP are required to comply with Regulation (EC) No 1107/2009. Therefore, before PPP are placed on the market, it should be demonstrated that they present a clear benefit for plant production and do not have any harmful effect on human or animal health (including that of vulnerable groups) or have any unacceptable effects on the environment.

Regulation (EC) No 1107/2009 applies to substances (referred to as 'active substances'), including micro-organisms, that have either general or specific actions against harmful organisms or on plants, parts of plants or plant products. The definition of micro-organism is: "any microbiological entity, including lower fungi and viruses, cellular or non-cellular, capable of replication or of transferring genetic material". To support the registration of PPP based on micro-organisms, there are adapted data requirements detailed in implementing regulations (EU) No 283/2013 for active substances and (EU) No 284/2013 for plant protection products. In addition there are guidance documents providing specific reference to micro-organisms available from OECD, SANCO and EFSA.

Bacteriophage as viruses can reasonably be considered as micro-organisms. They are not, however, specifically referred to in Regulation (EC) No 1107/2009 nor in the implementing regulations. Since the use of bacteriophage as PPP for agriculture and horticulture is a relatively new technology, the lack of guidance documents to allow interpretation of the data requirements in relation to bacteriophage is not unexpected (KIMURA, 2008).

This review considers how bacteriophage-based PPP can be regulated as a specific type of 'micro-organism', including consideration of approaches used in other OECD countries, the appropriateness of the current data requirements to this technology, as well as proposing how these data requirements may be addressed.

Box 2. Bacteriophage 'lytic' life cycle relevant to the control of plant microbial pathogens



*(**1**) Phage attaches to a precise host bacterium and (**2**) injects phage DNA, (**3**) interrupts the bacterial genome, killing the bacterium and captivates the bacterial DNA and protein synthesis machinery to make new phage parts. (**4**) The process finishes with the assembly of new phage, and (**5**) the lysis of the bacterial cell wall to release hundreds of new copies of the input phage into the environment.*

Relevant Commercial and Regulatory State-of-the-Art

State-of-the-art

By nature, bacteriophage are viruses that are capable of infecting only bacteria. Their extreme specificity means that they will not infect beneficial bacteria and also, they are not capable of infecting eukaryotic cells; i.e. they are not capable of infecting either animals, plants, or fungi. In a recent review of bacteriophage (KIMURA, 2008), the authors note that viruses cannot capture and store free energy and they are not functionally-active outside of their host cells. Furthermore, since they are not metabolically active, bacteriophage are incapable of producing any toxins outside their hosts. Animals safely ingest large numbers of bacteriophage in their diet and they have been used as therapeutic agents in humans for >80 years with no ill effects.

Taxonomy

Bacteriophage taxonomy is not simple and there is considerable debate about this subject (Nelson, 2004). According to (KIMURA, 2008) bacteriophage are classified by morphotype and host genus. Over 96% of bacteriophage are 'tailed' with linear, double-stranded DNA, belonging to the Order Caudovirales. This Order is divided into three families, based on morphology (tail type) and nucleic acid structure: Myoviridae with a contractile tail, Siphoviridae with a long non-contractile tail and Podoviridae with a short non-contractile tail (King, 2012). Siphoviridae are the most numerous and comprise 61% of the tailed bacteriophage.

Bacteriophage classification is determined by the International Committee of Taxonomy of Viruses (ICTV) & specifically the prokaryote virus sub-committee. Within the ICTV (http://www.ictvonline.org), bacteriophage classification is based on virion properties and nucleic acid, **not** on host range or pathogenicity (Box 3).

Virus taxonomy is being continually updated, with the latest definitive source published in 2012 (King *et al.*, 2012). Current virus taxonomy indicates that there are seven orders (containing 26 families) and an additional 77 families not assigned to an order. The ICTV, however, is considered to be far behind in its classification and this is particularly the case for bacteriophage since new bacteriophage are being discovered on a daily basis; hence, bacteriophage classification is always going to be open-ended, with the ICTV being significantly behind in its classification schedule. Many gaps exist; for example, more often than not, bacteriophage will be grouped within an order, family or sub-family based on similarities to other members but not be assigned to a genus.

Box 3. ICTV Definition of a virus species

According to the ICTV, species are created in accordance with the following definition: "A species is the lowest taxonomic level in the hierarchy approved by the ICTV. A species is a monophyletic group of viruses whose properties can be distinguished from those of other species by multiple criteria." The criteria by which different species within a genus are distinguished are established by the appropriate Study Group. These criteria may include, but are not limited to," natural and experimental host range, cell and tissue tropism, pathogenicity, vector specificity, antigenicity, and the degree of relatedness of their genomes or genes. The criteria used should be published in the relevant section of the ICTV Report and reviewed periodically by the Study Group."

Methods for Identification of Bacteriophage (Ackermann, 2011)

No universal method for viral (and hence bacteriophage) classification exists. The commonest approaches examine both morphology and nucleic acid composition. Morphological studies commonly rely on transmission electron microscopy to distinguish shape, presence/absence of tails, envelopes, features such as spindles/base plates and symmetry. This is usually the first approach to attribute bacteriophage to families.

Nucleic acids studies include:

a. Genome composition (ssRNA, dsRNA, ssDNA, dsDNA, linear or circular, molecular weight). This is generally non-specific for the classification of most bacteriophage as all of the tailed phage (Caudovirales) have double-stranded DNA. It can, however, confirm family designation.
b. Comparison of specific gene sequences e.g. polymerases or terminases; useful for some small groups but not across larger taxonomic groups and hence is considered unsuitable for identification.
c. Genome sequences: the commonest method, focussing on genome organisation, gene number, presence/absence/type of "modules" (genes required for a specific function e.g. replication, tail proteins, lysis genes) etc.

The complication here with bacteriophage identification is that the majority (70-80%) of bacteriophage genes are of unknown function and although a number of bacteriophage genome sequences are deposited in International Depositories, little other information is usually included with them.

Transfer of genes

The capacity for bacteriophage to transfer bacterial genes from one strain to another (transduction) varies from bacteriophage-to-bacteriophage. For example, RNA viruses cannot package and transduce DNA. Traditional methods to identify transducing bacteriophage rely on the transfer of genetic markers from a donor to a recipient strain (e.g. antibiotic resistance). Transduction frequencies vary according to the bacteriophage, genetic marker, bacterial donor and bacterial recipient used (Toth, 1997).

Information on bacteriophage genome structure can also be used to indicate the likelihood of potential gene transfer. For example genomes with fixed genome termini are unlikely to transduce, compared to bacteriophage genomes without cohesive ends of terminase recognition sites. The latter are likely to package DNA via a "headful" mechanism, with more than one full genome per bacteriophage capsid; which provides the capacity to transduce extra DNA. Bacteriophage packaging mechanisms can be inferred directly from genome sequencing reads or via nuclease restriction patterns (Loessner, 2000).

Biology and ecology

Bacteriophages are ubiquitous in the environment. For almost every bacterial species, there exists at least one bacteriophage that can specifically infect and ultimately destroy that particular bacterial group (Walker, 2006).

Following bacteriophage infection, lytic bacteriophage can either: i) take-over the host cell machinery or ii) replicate without affecting major metabolic pathways. In the case of i) the host cell take-over, host cell macromolecular synthesis is shut-off within a few minutes and bacteriophage replication begins. Host DNA is often rapidly degraded, preventing normal host gene expression and reducing the host to a "virus replication factory". In the case of ii) some bacteriophage can replicate whilst host gene expression and metabolism continues. The bacteriophage infection does alter gene expression patterns; however, the majority of virus-induced changes take place only after the synthesis of virion components, indicating that there is no major reprogramming of the host during early infection. The most highly-induced genes encode

chaparones and other stress-inducible proteins, with most metabolism genes showing no change in regulation (Poranen, 2006).

As with all bacteriophage, the exact bacteriophage/bacterial host combination (and environmental factors) will determine what happens during infection. However using lytic bacteriophage, most host bacterial gene expression is regarded as completely shut-off or largely unaffected by phage infection.

Bacteriophage are considered to comprise the majority among viruses in aquatic environments but this cannot be extended to soil environments, since fungal biomass is often larger than bacterial biomass in soils (KIMURA, 2008). Other recent reviews, however, indicate that bacteriophage density estimations range up to and over 10^8 g^{-1} in soil, with ratios of bacteriophage to bacteria in forested soils in the range of 10:1, whereas agricultural soils can have up to 10^9 g^{-1} bacteriophage (Abedon, 2011).

Commercial development

Bacteriophage-based biocontrol has great potential to enhance microbiological safety, based on their long history of safe use, relatively easy scale-up and handling and their highly-specific antimicrobial activity. Since their discovery in the early 1900's, bacteriophage have attracted interest as antimicrobial agents against human bacterial pathogens such as dysentery and they have been used in such therapeutic situation for over 80 years. This interest has been revived recently due to the threat of antibiotic-resistant bacteria and there are a number of commercially-available bacteriophage-based products targeting human diseases. Other areas of application include water and food safety (as biocides), crop protection and food processing and processing and animal health. In the area of food safety, the concept of combating pathogens in food by means of bacteriophage can be addressed at all stages of production in the classic 'farm to fork' approach throughout the entire food chain (Garcia, 2008) and indeed, bacteriophage have been used for over 10 years to control *Listeria* in meat, poultry and fish (outlined further below), (Walker, 2006).

Bacteriophages are highly specific, which is a commercial disadvantage when a disease is often caused by a large range of bacterial strains and frequently by different bacterial species. Commercial developments, however, are developing technological approaches to address this; for example this project's Contractor is working in the area of crop protection and food processing and is developing products for a range of applications within these sectors, including plant protection products (PPP).

The initial stage in developing a bacteriophage-based PPP involves the isolation of bacteriophage using enrichment techniques of a range of potential environmental sources (soil, sewage, plant material etc) with the host plant-pathogenic bacteria. For large scale production, bacteriophage are typically co-cultured with their bacterial host in liquid medium. Fermentation or other batch-culture systems are usually used, allowing the control of the relevant growth parameters for optimal production. Once produced, the bacteriophage are concentrated and purified; removing excess nutrients and the cell debris which remains following bacterial lysis. Individual bacteriophage are produced separately and if required, mixed in defined ratios to increase the product's efficacy against a wide range of bacterial strains.

The **specificity**, **safety** and **biodegradability** of bacteriophage make them the perfect 'natural biocontrol agents' and there is a significant market opportunity to develop effective bacteriophage-based PPP, given the increasing interest in biocontrol technologies and legal requirements for growers to integrate IPM into primary production. Commercial developments, however, face both technical barriers of accommodating bacterial variation on both a geographical and temporal level, in addition to potential freedom-to-operate (FTO) issues given the relatively large amount of prior art. There is a substantial amount of prior art in the area of using bacteriophage to control a wide range of bacterial pathogens, including those of plants. From the IP audit carried out as part of this study, key patents to consider possible potential FTO issues are: (i) "Bacteriophage T10-1, MY-1, and Vegetable Soft Rot Controlling Composition Containing the Same" – Republic of Korea KR20130020710, 2013. Whilst mention is made of targeting *Pectobacterium* spp. (one of the key agricultural plant pathogens), there are no foreign equivalents and FTO issues would only arise in Korea with the named bacteriophage. (ii) "Bacteriophage Treatment for Reducing and Preventing Bacterial Contamination", Ecolab Inc, US2009246336A, 2009. This patent describes the use of bacteriophage to prevent the spoilage of foods, including vegetables, although primarily during processing. As a US patent, FTO issues would arise in the US only. (iii) "Methods for Introducing Bacteriophage into the Interiors of Quantities of Edible Agricultural Products and Edible Agricultural Products with Phage-Containing Preparations in the Interiors Thereof", Omnilytics Inc, US2010068185A, 2010. FTO: a general disclosure only of the use of bacteriophage in methods of reducing bacterial contamination in food. The patent focusses on applying bacteriophage internally, mainly to food of animal origin. (iv) "Method of Treating Food Products", Omnilytics Inc., US2007292395, 2006, with separate territory filings (Australia, Canada, Japan, Europe). This is a highly specific patent, detailing lytic viral mutants for controlling pathogenic bacteria, including on plants, although FTO issues appear unlikely due to the specific nature of the claims. There is also some non-patent published literature that makes some

relevant general disclosures and inventive steps which potential commercial developments need to be aware of.

Regulatory precedence for bacteriophages

In the EU, EFSA has recently completed a comprehensive review of bacteriophage technology for use in food of animal origin including animal carcases, meat products and dairy products (EFSA, 2009, 2012). The principal debate in the EU centres around whether bacteriophage are able to prevent recontamination (and hence, would require to be registered as a preservative under the food additive classification rather than a processing aid) or not. EFSA's BIOHAZ Panel concluded that under specific conditions, bacteriophage may be very effective in the elimination of specific pathogens from foods. However, based on data currently available in peer-reviewed scientific literature, the Panel could not conclude whether bacteriophage can protect against bacteria in case the food becomes re-contaminated. Conclusions for this EFSA report are provided in Box 4. In addition, some relevant conclusions of this review were that "Bacteriophage in the environment behave as inert particles and tend to persist longer than their hosts. However, their long-term antibacterial activity is compromised on dry surfaces." and "The persistence in/on food varies with each bacteriophage, and with the conditions of application, including dose and physical and chemical factors associated with the food matrix."

The primary bacteriophage product on the market for use on food is Listex^{TM}, marketed by Microeos Food Safety (The Netherlands). Listex^{TM} is based on P100 bacteriophage and is used as a processing aid against a broad range of *Listeria monocytogenes* strains during the production of meat, cheese, fish, vegetables and other food products. The product is marketed in The Netherlands as a processing aid (positively reviewed by 'The Netherlands Organisation for Applied Scientific Research' (2010) and approved by the Dutch Ministry of Health) and is beginning to gain worldwide approval as a processing aid, including approval by the USDA and Health Canada, in addition to Food Standards Australia/New Zealand (FSANZ) and the Swiss Bundesamt fur Gesundheit**.** Listex^{TM} is also listed by the Organic Materials Review Institute (OMRI) in the USA and has been approved as a processing aid in organic food products by SKAL, the designated Public Inspection Authority of The Netherlands. In the USA, Listex^{TM} is granted GRAS status (GRAS 198) and the safety assessment notes that "There are more individual bacteriophages in the biosphere than there are of any other group of organisms, including all the prokaryotes." and "Numerous papers attest to the fact that humans are exposed to huge numbers of bacteriophage daily, through food and water, without notable evidence of any harm." However, a recent review by EFSA was unable to conclude that in the EU the same Quality and Safety Principle can be applied to bacteriophage. Furthermore, within EFSA's scientific opinion on the safety and efficacy of Listex P100 for removal of *Listeria monocytogenes* surface contamination of raw fish, a lack of data was noted a lack of data for certain studies, concluding that "Tests to investigate potential development of resistance or reduced susceptibility to biocides and key therapeutic antimicrobials, following use of Listex^{TM} P100, are recommended. The continuous effectiveness of Listex^{TM} P100 against *L. monocytogenes* and the potential for selection and dominance of strains naturally-resistant to P100 should be monitored." (EFSA, 2012).

EFSA's focus to date has been on the application of bacteriophage to food for combating human pathogens, mainly on food of animal origin, although also on vegetables in relation to *Listeria*. There has been no published consideration of the technology's potential for controlling bacterial pathogens of plants and plant products, either as processing aids or plant-protection products. Bacterial diseases can be a significant hindrance to the agricultural production of high quality, disease-free produce, with the effects manifested throughout the supply chain and in the final retail products. Bacteriophage technology offers a significant potential in this area but the uncertain regulatory path has a potential inhibitory influence and requires attention by the regulatory authorities.

Box 4. Conclusions relating to the mode of action expected from the use of bacteriophage solutions on food of animal origin (including but not exclusively use on animal carcases, meat products and dairy products). Terms of Reference number 1.

- Bacteriophage may be virulent or temperate. Upon infection, the first group kills their host bacteria, so they are the ones of choice for bacteriophage-based food decontamination.
- Temperate bacteriophage do not always kill their hosts, and may confer unforeseen properties to their host bacteria.
- Bacteriophage can induce lysis of the bacterial host-cell by "*lysis from within"* and/or "*lysis from without"*.
- Bacteriophage have narrow host-ranges, generally restricted to either a limited number of species within a genus, or to a limited number of bacterial strains within a species.
- While bacteriophage replicate best on growing bacterial cells, they have also been shown to reproduce on stationary-phase cells.
- The ratio of bacteriophage to host cells is critical to the success of bacteriophage treatment. The higher this ratio, the greater the reduction in the target bacterial population.
- Naturally-occurring bacteriophage have a broad range of habitats and may be isolated in considerable numbers from meat, milk and products thereof.
- Some bacteriophage, under specific conditions, have been demonstrated to be very effective in the targeted elimination of specific pathogens from meat, milk and products thereof.
- Bacteriophage-insensitive mutants might exist among the populations of target bacteria. The frequency of these mutations and their consequences are likely to vary according to the bacteriophage, the conditions of its application and the target bacteria.

The only examples of bacteriophage being developed and registered as PPP are the Agriphage™ range of products produced by Omnilytics Inc. (Utah, USA), with registrations in both the USA and Canada:

Registering authority	Product name/producer	Target disease(s)	Target pathogens	Registration details
EPA	Agriphage ™ (Omnilytics Inc., USA)	Bacterial spot of tomatoes & peppers Bacterial speck of tomatoes	*Xanthomonas campestris* pv. vesicatoria & *Pseudomonas syringae* pv. tomato	67986-1 (Dec 2005, amended June 2006 & Oct 2011)
EPA	Agriphage CMM (Omnilytics Inc., USA)	Bacterial canker of tomatoes	*Clavibacter michiganensis* sub sp. *michiganensis*	67986-6 (Sept 2011)
PMRA Canada	Agriphage CMM (Omnilytics Inc., USA)	Bacterial canker of tomatoes	*Clavibacter michiganensis* sub sp. *michiganensis*	RD2012-21 (Jan 2012)

Both the EPA and PMRA have followed a similar route in determining the suitability of bacteriophage for plant protection, particularly concerning their apparent safety and very low toxicity profiles, based both on data published in peer-review articles and submitted by the applicant. Indeed, based on these data EPA granted an exemption from the requirement of a tolerance for residues of Agriphage CMM bacteriophage in or on tomato when applied as a bactericide in accordance with good agricultural practices, concluding that "there is a reasonable certainty that no harm will result to the U.S. population from aggregate exposure to residues of the lytic bacteriophage of Clavibacter michiganensis subspecies michiganensis produced in Clavibacter michiganensis subspecies michiganensis." Similar conclusions were reached by the PMRA in their January 2012 decision on the product (Box 5) and these conclusions follow on from previously-published work (Walker, 2006) indicating that:

- bacteriophage are not capable of producing any toxins outside of their hosts because they are not metabolically active;
- bacteriophage rely on the bacterial host's metabolism for reproduction and survival;
- bacteriophage themselves are not considered to be toxic. They do not harm human or animal cells.

Box 5. Conclusion of PMRA review of bacteriophage of *Clavibacter michiganensis* (subsp. *michiganensis*)

By nature, bacteriophage are viruses that are only capable of infecting bacteria. Bacteriophage are not capable of infecting animals, plants, or fungi and are not capable of producing any toxins outside their hosts because they are not metabolically active. Bacteriophage rely on the bacterial host's metabolism for reproduction and survival. Bacteriophage themselves are not considered to be toxic. Also, since the host bacterium, *C. michiganensis* subsp. *michiganensis*, does not produce toxins nor is it otherwise considered to be harmful to humans, the infection of these bacteria by bacteriophage of *Clavibacter michiganensis* (subsp. *michiganensis*) will not alter the bacterial population in a way that could be harmful to humans. Although the relative exposure of people to bacteriophage of *Clavibacter michiganensis* (subsp. *michiganensis*) may increase from the use of AgriPhage-CMM, there have been no reports of adverse effects or incidents resulting from the direct exposure to naturally occurring bacteriophage.

MAIN IMPLICATIONS OF THE FINDINGS

Proposal for a regulatory approach for PPP in the EU

Regulation (EC) No 1107/2009 applies to substances, including micro-organisms having general or specific action against harmful organisms or on plants, parts of plants or plant products, referred to as active substances. The definition of micro-organism is: "any microbiological entity, including lower fungi and viruses, cellular or non-cellular, capable of replication or of transferring genetic material".

Bacteriophage as viruses can reasonably be considered as micro-organisms. However, bacteriophage are not specifically referred to in Regulation (EC) No 1107/2009 nor in the implementing regulations. The closest guidance document available for bacteriophage, because they are a type of virus, is the 'Guidance document on the assessment of new isolates of baculovirus species already included in Annex I of Council Directive 91/414/EEC' (SANCO/0253/2008 rev. 2). In this SANCO document, baculoviruses are considered a special regulatory case because:

1. Baculovirus species are extremely host-specific, with their host range limited to one or a few species of the same genus. Larger host ranges covering different genera or even different families are rare (e.g. *Autographa californica* NPV). Baculoviruses probably represent the most specific pesticidal agents, biologicals and chemicals taken together.
2. Baculoviruses occur only in arthropods, predominantly in the insect orders *Lepidoptera*, *Diptera*, and *Hymenoptera*.
3. Baculoviruses are not infective for mammals and replication does not occur in mammalian cells.
4. No pathogenic, genotoxic, mutagenic, or carcinogenic effect of baculoviruses has ever been observed in mammals.
5. Baculoviruses do not produce metabolites since they have no independent metabolism.
6. Effects on non-target species can be excluded, especially for vertebrates, micro-organisms, and plants.

Based on these principles, the question is whether a similar approach is reasonable for bacteriophage? If so, related to the qualifying criteria above:

1. Bacteriophage are extremely host specific, with host range limited to one or a few species of the same genus or often, one or a few strains of the same species. Larger host ranges covering different genera are rare. Bacteriophage probably represent the most specific pesticidal agents, biologicals and chemicals taken together; potentially more specific that baculoviruses.
2. Bacteriophage are ubiquitous but are specific to bacteria.
3. Bacteriophage are not infective for mammals and replication does not occur in mammalian cells.
4. No pathogenic, genotoxic, mutagenic, or carcinogenic effects of bacteriophage have ever been observed in mammals.
5. Bacteriophage do not produce metabolites since they have no independent metabolism.
6. Effects on non-target species can be excluded, especially for vertebrates, micro-organisms, and plants.

Summary and Conclusions

The close comparability of the criteria that make baculoviruses a special regulatory case with the same features of bacteriophage indicate that a similar regulatory approach can reasonably be adopted for bacteriophage. This would necessitate a number of adaptations of the data requirements as listed in SANCO/0253/2008 rev. 2. Suggested adaptations will be detailed in the Strategy Document for discussion with appropriate regulators and other bodies. The main areas where adaptations are suggested include:

1. Identity: since the current classification for bacteriophage has significant gaps and the morphological methods are neither practical or relevant, it is suggested that identity of the A.S. should include the taxonomic classification of the host bacterium, supported by molecular sequencing of the bacteriophage alongside its morphological grouping, with both bacteriophage and host bacterium being deposited in an internationally-recognised culture collection.
2. Residue studies: since bacteriophage are ubiquitous and no incidences of adverse effects have been identified, it is suggested that no maximum residue levels (MRL's) are required and hence, no methods needed to determine/quantify residues.
3. Toxicology: it is suggested that the requirements for toxicology studies are waived given the published scientific studies of the extremely low-risk profile of bacteriophage, their inability to infect mammalian cells and the lack of toxin production by bacteriophage. Similarly, negligible or no risk should be expected for the general public through dietary exposure to any bacteriophage residues and hence, such studies are also not required.
4. Environmental fate: the ubiquitous nature of bacteriophage and the lack of significant toxicological or ecotoxicological effects indicate that no fate data should be required to complete an environmental risk assessment of bacteriophage-based PPP.

FUTURE WORK

The principal outcome of the project is a proposed bacteriophage registration strategy document, which will have the potential to act as a basis for the OECD Biopesticide Steering Group, CRD, Defra and any other relevant authorities to discuss regulation of bacteriophage as biopesticides. This document, prepared by Rationale Biopesticide Strategists has not been included in this report since it still requires discussion with the relevant authorities prior to publication. We intend to make this document and related knowledge available to the above groups for discussion.

References to published material

9. This section should be used to record links (hypertext links where possible) or references to other published material generated by, or relating to this project.

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