

# The Bacteriophage in Biology, Biotechnology and Medicine

BioPark, Hertfordshire, UK: 26<sup>th</sup> Feb 2010

After our successful *Bacteriophage Applications - current and potential applications in biotechnology, agriculture and medicine* event which took place 16th May 2006 and *Bacteriophages: Nature and Exploitation* event which took place in 22 February 2008 we are delighted to announce our follow up event.

"Bacteriophages are the most abundant biological entities on earth and are major drivers of bacterial adaptive evolution. Studies on phage biology underpin our core knowledge of modern molecular biology - and phage research has provided many of the techniques and biochemical reagents used by all molecular biologists. This meeting is the third in a successful biennial series discussing the biological nature of bacteriophages, and their exploitation in basic microbiology (for genetics and functional genomics); diagnostics, ecology and evolution, phage display, vaccines, and in therapeutics in animal and human infections. The general structure of the meeting is to have short, expert presentations on this spectrum of topics, plus short Q&A sessions and offered posters. The meeting will be of interest to anyone who is currently using phages in their research work or who might be interested in the potential application of phages for basic biology, and applied topics such as bacterial diagnostics, vaccine development and phage therapy". Meeting chair -  
*Professor George Salmond, University of Cambridge, UK*

This event has CPD accreditation and will have a panel discussion session and you can submit your questions online during registration

8:30 - 9:00      **Registration**

9:00 - 9:10      **Introduction by the Chair: The nature and uses of bacteriophages**

*Professor George Salmond, University of Cambridge, UK*

9:10 – 9:40      **The link between pathogen evolution and bacteriophage**

*Dr Nicholas Thomson, The Wellcome Trust Sanger Institute, Cambridge, UK*

It is clear from genome sequencing that phage in recent times are probably the most significant source of variation for bacterial genomes. We have also seen that phage have had a long term impact on the virulence potential and evolution of many bacterial pathogens. Presented will be several case studies looking at the impact of phage on host variation and where we have used prophage to track the succession of different dominant Salmonella clinical isolates. Also described will be a study focussed on *Citrobacter rodentium* where phage have been significant in the evolution of this contemporary pathogen.

9:40 – 9:55      **NanoTracking Analysis (NTA) for Phage Material Characterisation, Sizing and Counting**

*John H Creedy, Progressive Research Systems Ltd, UK*

Despite the importance of obtaining accurate estimates of size, size distribution and concentration of viruses and bacteriophage in vaccine manufacture, existing methods for their quantification frequently rely on slow and complex techniques such as plaque assay or estimation of TCID. Nanoparticle Tracking Analysis (NTA) is a newly developed method for the direct and real-time visualisation and analysis of nanoparticles, such as viruses, in liquids. Based on a laser illuminated microscopical technique, Brownian motion of nanoparticles is analysed in real-time by a CCD camera, each particle being simultaneously but separately visualised and tracked by a dedicated particle tracking image analysis programme. Because each and every particle is visualised and analysed separately, the resulting estimate of particle size and particle size distribution is a direct number/frequency distribution which directly gives virus concentration. The ability of the NanoSight instruments to visualise, count and size viruses and their aggregates is becoming increasingly important to those people involved in the development of phage therapeutics and viral vaccines. Manufacturers are interested in monitoring the purity of the viral preparation at various key stages of the purification process and determine the concentration of virus material present. The particle-by-particle approach to sizing and counting viruses can easily distinguish viruses from larger cell debris and the high resolution number distributions obtained can be used to calculate the number of viruses vs. the number of virus aggregates. The total viral titre (i.e. infectious titre plus non infectious viruses) generated by the Nanosight technique can be used in conjunction with the infectious viral titre as provided by infectivity assays to understand what percentage of the total viral titre are infectious. In many cases the infectious viruses may represent as little as 1% of the total virus particles present in a preparation, and such low yield of infectious particles is perhaps indicative of the purification process used. As such, this information can be fed back into the process development to more effectively produce a final product.

9:55 – 10:25      **Phages from the sea: abundance and roles**

*Dr Martha Clockie, University of Leicester, UK*

Marine prokaryotic viruses are the numerically largest and most diverse group of organisms in the ocean. The vast nutrient poor oligotrophic areas of the ocean are dominated by cyanobacteria from the genera *Synechococcus* and

*Prochlorococcus*, and phages which infect them may be present at up to  $10^7$  per ml which is an estimated  $10^{30}$  phages in the ocean. Understanding the cyanobacteria-virus relationship in cyanobacteria is essential because marine viruses play a key role in the oceanic carbon cycle by causing lysis of a large proportion of the organic biomass on a daily basis and shunting nutrients between particulate and dissolved phases. During selective lysis of particular strains they shape cyanobacterial population structure. They are also a major source of horizontal genetic transfer and consequently a major driving force for the evolution and adaptation of cyanobacteria. Understanding cyanophages also has industrial relevance as they represent a valuable source of novel, commercially exploitable genes. This presentation will illustrate how insights from cyanophage genome studies have highlighted the complexity of the interaction between cyanophages and their hosts and directed downstream work to further understand this relationship.

10:25 – 10:30

**Speakers photo**

10:30 – 10:50

**Mid-morning break & poster viewing**

10:50 – 11:20

**Stx phages: the impact of multiple bacteriophage infections**

*Heather Allison*, University of Liverpool, UK

Shiga toxin encoding bacteriophages (Stx phages) have been the driving force behind the emergence of Enterohaemorrhagic *E. coli* (EHEC), Shigatoxigenic *E. coli* (STEC) and other Shiga toxin producing foodborne bacterial pathogens. Various molecular techniques have been used to demonstrate that these phages are a significant component of the viral community in the natural environment and encode a variety of additional factors that produce changes in the phenotype of their bacterial host. Characterisation of one particular Stx phage,  $\Phi$ 24B, has provided evidence that Stx phages can multiply infect a single host cell. And data will be presented to explain how multiple infection events occur. The impact that carriage of multiple Stx phages has on the host lysogen's toxin production potential and its further susceptibility to Stx phage infection will also be addressed. These findings emphasise that there is still much to learn about general bacteriophage biology, and illustrate the need to understand the role that bacteriophages play in directly altering the microbial ecosystem.

11:20 – 11:35

**Fast and Convenient Technology for Isolation and Purification of Phages using CIM Monoliths**

*Mr Franc Smrekar*, BIA Separation, Austria

Bacteriophages are a promising tool to deal with emerging health challenges. For majority of applications, a environment and high degree of purity are challenges that have to be overcome. Our aim was to investigate efficiency of bacteriophage concentration and purification using CIM monolithic support. We used T4, Lambda, T7 and M13 phages as a model system. Chromatographic methods using a linear gradient were implemented to investigate phage elution conditions and to establish the optimal chromatographic method applying step gradient. Finally, capacity and purity of all three phages was evaluated.

11:35 – 12:05

**Mechanism and applications of phiC31 integrase**

*Professor Maggie Smith*, University of Aberdeen, Scotland

The *Streptomyces* temperate phage, phiC31, encodes an integrase which is required for integration into and excision from the host chromosome. phiC31 integrase is a member of the serine recombinases, mechanistically and evolutionarily distinct from the lambda integrase family. We showed that the conditions required for integration by phiC31 integrase are very simple with no topological constraint, no requirement for an accessory factor and simple recognition sites. Moreover the recombination is unidirectional. These properties prompted the application of the phiC31 integration system as a general tool for genome engineering and it is a promising candidate for gene therapy in humans.

12:05- 12:35

**Fermentation of M13 and Lambda bacteriophages**

*Professor John Ward*, University College London, UK

Research and commercial interest into bacteriophages has increased as the number of potential uses in therapy and synthetic biology has been recognised. Techniques used for the production of bacteriophage at small-scale are not transferable to large-scale production in industrial processes. We have investigated the MOI of M13, the timing of addition of M13 and the titre of the host needed in fermentations.

For lytic phage such as lambda, nucleic acids are released and become a contaminant that has to be removed at later stages. An *E. coli* that secretes a nuclease has been developed and this obviates the need to add enzymes such as Benzonase™.

12:35 – 13:05

**Phage display technology and its applications**

*Dr John McCafferty*, University of Cambridge, UK

The display of antibodies on the surface of filamentous phage has proven to be a powerful technology in both research and drug discovery. Using phage display, libraries of antibody gene fragments are fused to the gene encoding a minor coat protein. This gives rise to a population of phage particles each displaying an antibody on the surface with the encoding gene contained within the particle. It becomes possible to isolate antibody genes encoding desired properties, by panning such repertoires on immobilised antigen. At Cambridge Antibody we used this

technology to generate Humira, a TNFalpha neutralising antibody, which is the first approved drug from this technology. The presentation will describe my current work using an antibody phage display library of 10 billion clones to isolate antibodies for a range of applications.

13:05 – 14:00 **Lunch and poster viewing**

14:00 – 14:30 **Question and Answer Session**

Delegates will be asked to submit questions to a panel of experts. Questions can be submitted before the event or on the day

14:30 -14:45 **Recent Advances in Phage Vaccination.**

*Dr Ewan Clark, Big DNA Ltd, Scotland, UK*

We have used bacteriophage lambda as a DNA vaccine delivery vehicle. We have tested several reporter and vaccine antigens in a number of animal species (mice, rabbits, sheep and fish). In most cases, the phage vaccine works more effectively than naked plasmid vaccination and in some cases is more effective (as measured by antibody responses) than recombinant protein vaccines.

The practical considerations involved in developing phage vaccines as a product, including thoughts on proceeding to phase I clinical trials with an injectable phage product in humans, will also be discussed. We are currently designing an optimised lambda vector which will be completely synthesised and will include several safety features and optimisations to improve yield. We are also developing novel purification methods, which meet the rigorous standards required for producing a product for human use.

14:45 – 15:15 **Exploiting phage technologies in the food industries**

*Dr Cath Rees, University of Nottingham, UK*

15:15– 15:45 **Afternoon Tea and last poster viewing**

15:45– 16:15 ***Uses of phage in the animal food production chain***

*Professor Ian Connerton, University of Nottingham, UK*

Interest in the potential of bacterial viruses to treat bacterial infection (phage therapy) is on the increase due to the quest to find alternative, sustainable methods to replace antibiotic treatments which no longer perform due to the dramatic rise in multi-drug resistant bacteria. This form of intervention is attractive for use with farm animals as not only is the application of phage potentially sustainable, their ubiquitous ensure we are not adding any thing to the food chain that is not already there and to which the consumer has not already been exposed. Moreover the specificity of phage treatments makes them an attractive proposition for the biosanitisation of meat products including ready to eat foods. The presentation will review the applications of phage in the animal production sector and examine the theoretical basis for these applications based on kinetic models of phage-host bacteria interactions.

16:15 – 16:45 **Acute wound infection control with bacteriophages**

*Dr Janice Spence, Glasgow Biomedical Research Centre, Scotland*

Phages immobilised by corona discharge technology onto sutures were used to secure an experimental wound. Wounds were infected with various concentrations of EMRSA 15 and the immobilised sutures used both to secure and close the wound. Control wounds showed little healing and clinical signs of infection whereas the phage coated sutures resulted in apparently normal healing. Histological examination confirmed the normal progress of the phage treated wounds. Microbiological examination showed some residual infection in all wounds, though less in phage treated wounds.

16: 45 - 17:00 **Summing up by the meeting chair**

You can network with people from this event at

**Nature network** - <http://network.nature.com/groups/euroscicon/>

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**Twitter** - <http://twitter.com/Euroscicon/>

*This meeting was **organised by Euroscicon** ([www.euroscicon.com](http://www.euroscicon.com)), a team of dedicated professionals working for the continuous improvement of technical knowledge transfer to all scientists. Euroscicon believe that they can make a positive difference to the quality of science by providing cutting edge information on new technological advancements to the scientific community. This is provided via our exceptional services to individual scientists, research institutions and industry. The event was hosted by **'BioPark** ([www.biopark.co.uk](http://www.biopark.co.uk)), a research and development centre in Welwyn Garden City providing specialist facilities and support for bioscience and health technology businesses to grow, and to develop new products and technologies*

### About the chair

**Professor Salmond** is currently in the Department of Biochemistry at the university of Cambridge. He graduated in microbiology (BSc, Strathclyde) followed by a PhD in bacterial genetics and phage-host interactions (Warwick) and an MA and ScD (Cambridge). He has taught in Kent, Warwick and Cambridge universities. He has multiple research interests in microbiology, including the molecular basis of bacterial virulence in plant and animal pathogens, quorum sensing, biosynthesis and regulation of bioactive secondary metabolites (including antibiotics), protein secretion systems, and the biology and exploitation of bacteriophages - the subject of this meeting. He has long-standing interests in the isolation of novel phages from the natural environment for the development of genetics and functional genomics of diverse bacteria, including plant, animal and human pathogens. He also has current research interests in how bacteria evade the potentially lethal impacts of viral infection via phage abortive infection systems.

### About the Speakers

**Martha Clokie** obtained a BSc in Biology from the University of Dundee followed by an MSc from the University of Edinburgh in Plant Biodiversity, and a PhD from the University of Leicester in Molecular Ecology. She completed two Post-Docs at University of Warwick working on horizontal gene transfer and cyanophages and subsequently characterising the contribution of host encoded cyanophage genes to cyanobacterial physiology. She spent four months as a visiting scholar at Scripps, La Jolla before starting a research group at the University of Leicester in September 2006. Her interests are focussed on the ecology and molecular biology of bacteriophages and their relationship with bacterial hosts; ranging from cyanobacteria to bacterial pathogens. She is also interested in exploiting bacteriophages and phage-derived products as an alternative to treating antibiotic resistant bacterial infections.

**Franc Smrekar** is a PhD Candidate at the University of Ljubljana. He is working in R&D department at BIA Separation, company that is manufacturing chromatographic columns for purification of biomolecules. He is working on the field of DNA, human virus and bacteriophage purification.

**John Ward** studied Biochemistry at the University of Bristol and moved to the Department of Bacteriology in Bristol for a PhD on antibiotic resistance plasmids and transposable elements. A postdoc in UMIST, Manchester working on *Pseudomonas* catabolic plasmids followed and in 1983 he moved to University College London. His current research is in oral metagenomics, phage display, bicatalysis and synthetic biology. He was appointed Professor of Molecular Microbiology in 2006.

**Janice Spencer** graduated with a BSc (Hons) in microbiology from the University of Glasgow and moved to London to complete her PhD studies at the Central Public Health Laboratory in Colindale. She continued her research career with post-doctoral studies at the University of Glasgow Veterinary School and the University of Strathclyde. She has also worked for Oxoid and Blaze Venture Technologies. She is currently working at the University of Glasgow investigating vaccine strategies for *Clostridium difficile*. Her main interests are the prevention of bacterial disease and protection against pathogens.

**Ewan Clark** graduated in 2004 from Edinburgh University with an honours degree in biotechnology. He has since completed a PhD project followed by a year working as a Post-Doctoral Researcher in the division of bacteriology at Moredun Research Institute. During summer 2009 the opportunity arose to join BigDNA Ltd., a company founded on the back of novel technology applicable to the delivery of DNA vaccines using bacteriophages. Ewan's position as a Research and Development Scientist requires him to design and evaluate DNA vaccine constructs using practical molecular biological techniques as well as coordinating phage and *E. coli* DNA sequencing projects.

**Ian Connerton** started academic life began at the University of Warwick where he studied Biochemistry before completing Ph.D. studies in the Chemistry Department through an industrial case award. He spent three years as a research assistant in the Genetics Department at Cambridge University working on the molecular genetics of filamentous fungi. He left Cambridge in 1987 to join the Microbiology Department at Reading, where he began to teach Food Science students and to appreciate the utility of broad academic knowledge to undertake multidisciplinary research in this area. Professor Connerton joined the Institute of Food Research in 1991 first as a Section Leader and graduating to Deputy Head of Food Macromolecular Science with Institute Programme responsibility for Macromolecular Function and Design. Prof. Connerton was appointed as the first Northern Foods Professor of Food Safety at the University of Nottingham in 1998. The post has required liaison with the members of the food industry, governmental organisations, and other research scientists, as well as interacting with representatives of various sectors of the media. At Nottingham Prof. Connerton has developed research collaborations on the host pathogen interactions of the food borne bacterial (*Campylobacter*, *Salmonella* and enterovirulent *E. coli*) and viral pathogens. These include collaborations with clinical colleagues at the Queens Medical Centre, where he has become a member of the Centre for Digestive Disease at Nottingham. Prof Connerton will speak on the use of bacteriophages to control bacterial pathogens in animal production.

**Nick Thomson** began his academic career at Warwick University with a degree reading Microbiology and Microbial technology. Staying on at Warwick he went on to complete a three-year PhD in bacterial molecular biology. The focus of his studies was the production and regulation of secondary metabolites. For his Post Doctoral work Nick moved over to the Biochemistry Department at Cambridge University where he focussed on global regulatory systems in an opportunistic human pathogen, *Serratia*, and the phytopathogen, *Erwinia*. He then joined Pathogen Genomics (formerly Pathogen Sequencing Unit; PSU) at the Wellcome Trust Sanger Institute, as a Senior Computer Biologist, and is currently a Senior Staff Scientist for bacterial whole genome sequence projects of many important pathogens, principally the 'enterics' and the Chlamydia, but his work also includes analysis of bacterial viruses.

**Maggie Smith**: BSc University of Leeds (Biochemistry and Microbiology), PhD University of Bristol (Bacteriology), Post-Doc Leeds and Glasgow, Lectureships Stirling and Nottingham, Reader and Chair Nottingham, Chair Aberdeen

**Mr John Creedy** established Progressive Research Systems Ltd, (PRS Ltd) in 1990 as a vehicle for promoting innovative developments in purification technology. High impact innovative technologies are identified which offer key advantages in downstream processing of biological products including proteins, vaccines and virus related products. John has a background in bioseparations technology with LKB Instruments Ltd, Pharmacia Biotech (now GE Healthcare), and TosoHaas GmbH (now Tosoh Bioscience) in both analytical separations and process manufacturing scale chromatography. PRS Ltd currently acts as a promotional and marketing agency with BIA Separations GmbH, Atoll GmbH and NanoSight Ltd to identify new market sectors and introduce customers to these enabling technologies.

**John McCafferty** was one of the founders of Cambridge Antibody Technology (now Medimmune) in 1990 and was principle inventor of antibody phage display, a powerful technology for generating human antibodies. An antibody derived from this technology is now an approved product for rheumatoid arthritis with worldwide sales in 2007 of over \$3 billion dollars. A variety of others phage derived antibody products are in clinical and pre-clinical stages. After 12 years at CAT, John set up a group at the Sanger Institute developing and utilising methods for protein generation and recombinant antibody isolation in high throughput for research applications. McCafferty currently runs a laboratory at the Biochemistry Dept at University of Cambridge capitalising on the above technologies with a focus on the study of protein:protein interactions driving direct cell:cell communication.

## POSTERS

### FERMENTATION OF M13 AND LAMBDA BACTERIOPHAGES

E. Stanley, S. Branston, E. Keshavarz-Moore and J. Ward

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Bacteriophage M13 and lambda are well characterised viruses which over many years have been the workhorses of molecular biology laboratories. In recent years, research and commercial interest into these bacteriophages has increased as the plethora of potential uses in the pharmaceutical market and synthetic biology has been recognized. These include therapeutics against pathogenic bacteria, delivery vehicles for protein and DNA vaccines, delivery of gene therapy vectors and uses as structured scaffolds in synthetic biology applications such as electronics. Many techniques used for the growth and purification of bacteriophage at small-scale are not transferable to large-scale production of bacteriophage in industrial processes. In our studies high titre M13 production in *E. coli* has been optimised for 2 L fermentation conditions (1.5 L working volume). The final titre of M13 produced during the optimisation studies was independent of the multiplicity of infection used, however the time required to achieve the maximum titre of M13 was dependent on the MOI. The titre of the starting *E. coli* inoculum covering two orders of magnitude, was found to have no affect on the final yield of progeny M13 produced, however at the low *E. coli* numbers this increased the fermentation time required to achieve the maximum M13 yield by 30 – 60 min. Production of M13 in a 2 L fermenter where *E. coli* and M13 were inoculated together at the start of fermentation produced a maximum M13 yield of  $2.25 \times 10^{12}$  pfu/ml. However, delaying the addition of the M13 inoculum into the fermenter to 3 h post *E. coli* inoculation reduced the maximum M13 yield to  $4.58 \times 10^{11}$  pfu/ml. Initial fermentation studies for lambda production have concentrated on both the high titre production of lambda bacteriophage but also on overcoming several of the difficulties of large-scale lambda down-stream processing. An *E. coli* host was genetically engineered to produce a non-specific nuclease which is exported to its periplasm. Upon lysis of the *E. coli* by lambda the nuclease is released into the surrounding media and successfully degrades the *E. coli* DNA and RNA. The use of this engineered host removes the need to add bovine DNase/RNase or Benzonase™ when producing pharmaceutical grade lambda bacteriophage in a fermenter. The production of lambda in both a non-nuclease producing parent *E. coli* strain and the nuclease producing strain have been compared in 2 L fermenter (1.5 L working volume). No detrimental effect on the *E. coli* titre and growth profile was observed between the two strains.

### PREVENTION OF *CLOSTRIDIUM DIFFICILE* TOXIN PRODUCTION USING BACTERIOPHAGE THERAPY, IN AN *IN VITRO* COLON MODEL.

Emma Meader<sup>1,2</sup>, Melinda J Mayer<sup>1</sup>, Michael J Gasson<sup>1</sup>, Dietmar Steverding<sup>2</sup>, Simon R Carding<sup>1</sup> and Arjan Narbad<sup>1</sup>.

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*Clostridium difficile* is primarily a nosocomial pathogen, causing thousands of cases of antibiotic-associated diarrhoea in the UK each year. In this study, we used a continuous colon model system colonised with *C. difficile*, to evaluate the potential of a prophylactic bacteriophage treatment regime to control the pathogen. It is shown that the prophylaxis regime precluded the production of detectable levels of toxins A and B. The numbers of commensal bacteria including total aerobes and anaerobes, *Bifidobacterium* sp., *Bacteroides* sp., *Lactobacillus* sp., total *Clostridium* sp., and Enterobacteriaceae were not significantly decreased by this therapy. Our study indicates that phage therapy has potential to be used for the control of *C. difficile*; it highlights the main benefits of this approach, and some future challenges.

# MOLECULAR METHODS FOR THE STUDY OF FREE VIRUSES IN THE ENVIRONMENT

**D.J. Rooks, A.J. McCarthy and H.E. Allison**

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*Escherichia coli* (*E. coli*) is a member of the natural human gut microflora, but acquisition of various virulence factors can facilitate its transformation from a commensal organism to an overt pathogen. Conversion of a member of the enteropathogenic *E. coli* (EPEC) group to a hypervirulent enterohaemorrhagic *E. coli* (EHEC) was primarily due to the acquisition of the ability to produce Shiga toxin (Stx1 and Stx2), which was the result of phage conversion by temperate bacteriophages encoding Stx (Stx phages). Stx phages are diverse but share a distinct genome organisation with the archetypal phage, Lambda ( $\lambda$ ), and are therefore termed lambdoid. Ruminants are the primary reservoir of Shiga toxin producing *E. coli* (STEC), and it is likely that most cattle carry STEC in their gastrointestinal tract at some point in their lifetime, and these are shed in their faeces and transmitted to humans via contaminated food and water. STEC are a recognised significant public health concern worldwide causing diarrhoea, hemorrhagic colitis (HC) haemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) in humans. Although there are data on the distribution of STEC *i.e.* Stx phage lysogens in the natural environment, the occurrence and characterisation of Stx phage as free entities has received little attention, and consequently the epidemiological significance of Stx phages in the environment is unknown. Furthermore, it is likely that the phage population is underestimated in any given environment because of the limitations of traditional identification and propagation techniques. Consequently a phage detection and enumeration method that does not rely on the detection of host lysis *in vitro* would provide meaningful estimates of phage numbers and phage groups in environmental samples. The *stx* gene (the canonical marker for Stx phages), is located downstream of the anti-terminator gene *Q*, and although diverse in sequence, exhibit a degree of sequence conservation, which enabled the design of qPCR primers that can amplify all known *Q* and *stx* sequences. Application of these primer sets to water samples possessing no detectable *E. coli*-infecting phages by plaque assay, demonstrated that the number of lambdoid bacteriophages ranged from  $4.7 \times 10^4 \text{ mL}^{-1}$  to  $6.5 \times 10^6$ , with one in  $10^3$  free lambdoid bacteriophages carrying a Shiga toxin operon (*stx*). There is little or no information on the composition of the virome or viral metagenome, especially in freshwater habitats that have a role in virus dissemination. We have developed a novel method for harvesting viral DNA which does not contain any detectable cellular DNA by end point PCR, or by homology searches of a 454 pyrosequence derived metagenomic library against the RDP database. The pyrosequenced virome generated from a freshwater sample contained a total of 41, 916 sequence fragments (~224 bp), of which 24% were assigned an identity by BLASTX using the SEED database. We used qPCR to quantify the number of lambdoid and Stx phages in the sample, and the qPCR ratios were similar to the abundance ratios calculated from the 454 virome data. However, the absolute numbers of lambdoid and Stx phages obtained by qPCR suggest that the depth of sequencing performed had enabled us to examine only ca. 5% of the total virome. These methods, used alone and in combination, represent a novel approach to epidemiological/ecological studies on the occurrence, distribution and population dynamics of free viruses (*i.e.* not associated with an infected host cell) in the environment.

## BACTERIOPHAGE THERAPY: THE SOVIET EXPERIENCE

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Felix d'Herelle proposed that bacteriophage might be applied to the control of bacterial diseases, however in the West this idea was not explored with the same enthusiasm as in the former Soviet Union (FSU) and was eventually discarded with the arrival of antibiotics. The Eliava Institute of Bacteriophage, Microbiology and Virology (IBMV) was founded in Tbilisi in 1923 through the joint efforts of d'Herelle and the Georgian microbiologist, George Eliava. Due to the pursuant work of the staff of the Eliava Institute bacteriophage therapy was researched and applied extensively within the FSU for the treatment of a wide range of bacterial infections. Much of this work was published in Russian and was unavailable in the West. This fact inspired the authors to write a monograph ("A literature Review of the Practical Application of Bacteriophage Research", 2009, Tbilisi, Georgia, 184p) based on the historical publications found in the library of the Eliava Institute. The majority of the articles are from 1930s and 1940s, when this type of therapy was still new and experimental. Many authors described the methodology and results obtained and provided analyses and comments on the collected experimental data. Our analysis of the literature indicates that phage therapy was used extensively to treat a wide range of bacterial infections in the areas of dermatology, ophthalmology, pediatrics, surgery (especially against wound infections), urology, pulmonology, otolaryngology and stomatology. In this publication we present some examples of phage therapy and prophylaxis in surgery, the treatment of wounds and the treatment of intestinal infections to help stimulate further interest in its application and development at a time when "alternative" or "ordinary" therapies are becoming impotent in combating the increasing range of multi-drug resistant bacteria to which the phages still remain active. We present some examples of phage therapy and prophylaxis to help stimulate further interest in its application. It is also important that phage therapy may be considered as a method in combating the increasing range of multi-drug resistant bacteria to which the phages still remain active.

# IDENTIFICATION OF GENES DIFFERENTIALLY EXPRESSED IN *E. COLI* LYSOGENS OF THE SHIGA TOXIN ENCODING BACTERIOPHAGE $\Phi$ 24<sub>B</sub>

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The enterohaemorrhagic *Escherichia coli* (EHEC), including O157:H7, are food borne pathogens that have become a worldwide public health concern during the last two decades. Symptoms of EHEC infection range from mild diarrhoea to haemorrhagic colitis, which can be followed by one of two potentially fatal complications, haemolytic uraemic syndrome or thrombotic thrombocytopenia purpura. The major virulence determinant of EHEC is the production of Shiga toxin, which is encoded on temperate lambdoid bacteriophages (Stx-phages) that possess  $\lambda$  phage-like genomic organisation and regulatory functions. However, Stx-phage genomes can be as much as 50% larger, and many phage genes encode unknown functions. There are examples of bacteriophage  $\lambda$ -encoded proteins that enhance the survival of their lysogen, and we are identifying Stx phage gene products that might function similarly, especially as many are conserved across a range of otherwise heterogeneous bacteriophages. Proteomic techniques were used to identify genes differentially expressed by *E. coli* lysogens of  $\Phi$ 24<sub>B</sub>. Work with 2D-PAGE identified significantly different protein expression patterns between MC1061/ $\Phi$ 24<sub>B</sub>::Kan lysogen and naïve MC1061 cultures; further analysis with MALDI-TOF identified six differentially expressed proteins. A qPCR-based strategy was designed to confirm whether expression of these genes genuinely occurred in the *E. coli* lysogen population or was due to background levels of bacteriophage induction. Accordingly, we have demonstrated that the expression of at least 2 of these proteins is lysogen specific i.e. the phage genes are expressed *in situ* in the *E. coli* genome during normal growth. Work carried out using Change-mediated antigen technology (CMAT) has identified a further 16  $\Phi$ 24<sub>B</sub>-encoded genes that are expressed in lysogen cultures, though these have yet to be definitively associated with genuine *E. coli* lysogen expression profiles. Current work is focussed on the assignment of biological function to the proteins identified through the creation of recombinant proteins, protein assays and structural analysis. At least in the case of  $\Phi$ 24<sub>B</sub>, Shiga toxin production is not the only phenotypic change that *E. coli* undergoes following infection with Stx phage.

## MOLECULAR CHARACTERIZATION OF SALMONELLA BACTERIOPHAGES

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The gastro-intestinal infections as reported by National Centre of Disease Control of Georgia had outnumbered 2000 in years 2007-2008. The reported number of incidence of salmonellosis in Georgia is relatively small and corresponds to approximately 300 per year. However it is necessary to underline that the number doesn't reflect a real situation. The actual number of cases in the country may be higher. Unfortunately because of the poverty and lack of the health insurance system in Georgia people prefer self-treatment and apply to a doctor only in the very severe cases. Therefore the Disease Monitoring of the Health Care System can not enumerate all patients. Because the Federal Register of August 18, 2006, FDA announced that it had approved the use of a bacteriophage preparation constructed of six individually purified phage clones to be used on Ready to Eat meat and poultry products as an antimicrobial agent against *Listeria monocytogenes*, the salmonella bacteriophages could be used for sanitation of foods or feeds and may also be applied for therapeutic treatment outside the former Soviet and East European countries in which this type of treatment and prophylaxis has been established as traditional since early 1920-1930's. It should be underlined that the therapeutic bacteriophages are molecular less characterized or even uninvestigated. It would be rather important to investigate the phage genome for the presence of bacterial virulence genes which enhance the bacterial pathogenicity. Theoretically these genes could be horizontally transferred between bacteria and thus increase the probability of emergence of new highly pathogenic bacterial strains.

Sixteen new phages were isolated from different water sources in Georgia: River Mtkvari, Supsa, Chorokhi, Kvirila and sewage waters. To isolate new phages 18 different strains from our Laboratory collection have been used. At the beginning of experiments the most sensitive strains against the corresponding phages were used as the host strains. According to the host range and lytic activity seven of sixteen obtained phages were selected. Each phage was purified and concentrated to achieve high titers ( $10^8$ - $10^{10}$  pfu/ml). 120 strains related to Salmonella Typhimurium, Enteritidis, Bovinis, Agona, Infantis, Derby were used in the screening experiment. The strains were screened against seven newly isolated phages. The spectrum of lytic activity of the selected phages varies between 80% - 97%. To find out whether the selected phages absorb on a protein or polysaccharide receptor they were tested against the mutant strains missing one or more receptors (*fepA*, *iroN*, *cir*). High lytic activity against the mutant strains indicates that these phages usually attach to polysaccharide receptor. Corresponding primers were selected for different Salmonella prophages, the genomes of which are known and sequenced. The selected primers corresponded to the genes of the prophages P22, ST64B, ST64T, Gifsy 1, 2, 3, Fels1, 2 ES18 responsible for general transduction, integration and virulence of the bacterial strains and immunity to super-infection with relative phages. The results of PCR test have demonstrated that the phages BI2<sup>1</sup>, BI2<sup>2</sup>, BI2<sup>3</sup>, BI4, BI6 are similar to P22, and the phage BI3 stands closer to ST64B, while the phage BI1 is relative to ST64T.

Distribution of immunity genes (*imm* genes of ST64B and ST64T) among different phages indicated that these genes are conservative and the gene modules could be exchanged between various phages. The phages were also tested for carriage of virulence genes: Gifsy1 (*gogB*, *gipA*, *gtgA*), Gifsy2 (*sodC*, *sseI*), Gifsy 3 (*sspH1*), Fels1 (*nanH*), Fels2 (*conI*) Es18 (*gene1*, 2, 3). Lack of PCR products indicated that the phages BI1, BI2<sup>1</sup>, BI2<sup>2</sup>, BI2<sup>3</sup>, BI3, BI4, BI6 do not contain virulence genes and correspondingly are not related to the above mentioned prophages.

## DESIGN OF A LIPID NANOVESICLE SYSTEM ENCASING BACTERIOPHAGES FOR INHALATIONAL THERAPY: A PROOF-OF-CONCEPT.

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Inflammatory diseases that occur in the pharynx and involving both the adenoids and tonsils are important not only for being very frequent, but also because they often require minor surgery for their resolution. These structures have immunological functions leading to production of antibodies, and work in the local immunity of the pharynx and protection of the entire body. The most common etiologic agent of sore throats is *Streptococcus pyogenes*, an important pathogen of the beta-hemolytic group A which causes streptococcal pharyngitis. The emergence of antibiotic-resistant bacterial strains and the poor penetration of chemical antibiotics in bacterial biofilms raise the need for safe and effective options of antimicrobial treatment. The application of bacteriophages (or cocktails therefrom) has been proposed as an alternative (or complement) to conventional chemical antibiotics, allowing the release of natural predators of bacteria directly on these biofilms. The major advantage of bacteriophage-based antibiotherapy relative to its conventional chemical counterpart is that bacteriophages replicate at the site of infection, being available in abundance where they are needed the most. When compared with chemical antibiotics, bacteriophages have other important advantages: (i) strong tissue permeability, (ii) bacteriophage concentration remains high at the focus of infection, continuously increasing with bacterial (host) presence, (iii) elimination of the focus of infection occurs only after eradication of the host bacterium, (iv) bacteriophages are fully compatible with antibiotics and may act synergistically, (v) they are specific against the target bacteria, (vi) have a superior ability to penetrate bacterial biofilms, inducing production of enzymes that hydrolyze the biofilm polymeric matrix, (vii) although bacteria can develop resistance to bacteriophages, isolation of new lytic bacteriophages is much simpler and cheaper than developing a new chemical antibiotic. In this research effort, development of a biotechnological process for the inhalational administration of a bacteriophage cocktail (endotoxin free) was pursued, using strategies of nanoencapsulation within lipid nanovesicles (as forms of protection for the bacteriophage against the immune system) to treat infectious pathologies such as pharyngo-tonsillitis caused by *Streptococcus pyogenes*. This method of targeting may have a high potential for the treatment of bacterial infections of the respiratory tract, since inhalation therapy is considered to be favorable to certain respiratory infections because the aerosol is delivered directly at the site of infection, accelerating the action of bacterial predators. Additionally, a smaller amount of bioactive substance is needed, thus preventing or reducing possible side effects. As a proof of concept for the nanoencapsulation strategy, and since there is not yet available a strictly lytic bacteriophage cocktail for *Streptococcus pyogenes*, a well-defined and characterized bacteriophage was utilized, viz. bacteriophage T4. Water-in-oil-in-water (W/O/W) multiple emulsions are nanosystems in which dispersions of small water droplets within larger oil droplets are themselves dispersed in a continuous aqueous phase. Due to their compartmentalized internal structure, multiple emulsions present important advantages over simple O/W emulsions for encapsulation of biomolecules, such as the ability to carry both polar and non-polar molecules, and a better control over releasing of therapeutic molecules. T4 bacteriophage was entrapped within W/O/W multiple nanoemulsions, aiming at mimicking the multifunctional design of biology, optimized with several lipid matrices, poloxamers and stabilizing layer compositions. Physicochemical characterization of the optimized bacteriophage-encasing nanovesicle formulations encompassed determination of particle size, size distribution and particle charge, via Zeta potential analysis, surface morphology via CRYO-SEM, and thermal analysis via DSC, whereas antimicrobial activity of the nanoemulsions produced were evaluated via the "spot-test" using appropriate bacterial cultures.

### SAFETY CONCERNS REGARDING THE USE OF PHAGES IN ANIMAL FOOD PRODUCTION

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Phages have been proposed as natural antimicrobial agents for administration to living animals to ensure safe meat products. This is supported by several scientific studies, which demonstrate that phages are able to control the numbers of pathogens in animals. However, there are important safety issues that should be taken into consideration when developing a phage product to be applied to living animals. For example, it is of utmost importance to ensure that phages are strictly lytic do not encode bacterial toxins and do not recombine with cryptic phages encoded in target strains. The phage administration strategy and timing should also be adequate in order to reduce the development of phage-resistant bacteria. The present work describes the main strategies used in the development of a safe phage product for veterinary application, based on the results obtained on the scope of the European Project Phagevet-P (Veterinary Phage Therapies as Alternatives to Antibiotics in Poultry Production FP6-2003-Food-2-A:007224). Corresponding author: jazeredo@deb.uminho.pt

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