



Research Article

## Rapid Identification of *E. coli* Bacteriophages using Mass Spectrometry

Serafim V, Ring C, Pantoja L, Shah H, Shah A

### Abstract

**Objective:** The current increasing interest in the application of mass spectrometry, in particular MALDI-TOF MS, to identification of bacteria and fungi calls for a need to utilise this technology for identification of other infectious agents such as viruses. The aim of the present study was to develop a rapid and reliable mass spectrometry-based proteomic method for identification of *Escherichia coli* phages.

**Methods:** The approach was based on rapid in-solution tryptic digestion of suspensions of plaque-purified bacteriophage followed by mass spectral analysis. Matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS) and liquid chromatography – tandem mass spectrometry (LC-MS) were used to analyse the tryptic digests. Processing of tandem mass spectrometry data and interpretation of results were achieved using Mascot software and the Swiss-Prot database.

**Results:** Five bacteriophage species (Enterobacteria phages P2, T4, T5, T7 and Lambda) isolated in *E. coli* cultures were identified. The viral proteins were identified from a complex mixture of host bacterial proteins. In addition, using a single ion monitoring method, a Lambda prophage derived protein was also identified.

**Conclusion:** The data obtained demonstrate that LC-MS/MS can be used for accurate identification of *E. coli*- specific bacteriophages in both lytic and lysogenic cycles.

### Keywords

Bacteriophage virus; Mass-spectrometry; Liquid chromatography; MALDI; LC-MS/MS; Lytic; Lysogenic; Enterobacteria; *E. coli*; Phage; Viruses

## Introduction

The global interest in applying mass spectrometry (and MALDI-TOF in particular) for bacterial identification has resulted in the development of new identification methods which are fast, robust and relatively inexpensive [1]. A logical trend would be the transfer of this approach to virus detection and identification [2,3]. Using the profile of intact proteins proved very successful for bacteria identification and, in most cases, provides a species level identification [1,4]. However, this approach cannot be used for viruses because they possess a restricted range of proteins and, furthermore, they cannot

be produced in pure cultures. Therefore, the approach needs to concentrate on analysis of peptides obtained after tryptic digestion of viral proteins.

Bacteriophages ('phages') are viruses that infect and replicate within bacteria. Many consist of a head (capsid) and a tail-like structure. The capsid encases the genetic material and is formed by multiple copies of one or a few proteins, most often having an icosahedral structure. The genome (DNA or RNA) may encode from a few to as many as hundreds of genes. The tail-like structure is responsible for attaching to the bacterial wall and delivering the phage genome [5,6].

Once inside the host cell, bacteriophages may replicate and destroy the cell (lytic cycle) or remain in a dormant state in which the phage nucleic acid becomes incorporated into the bacterial genome (lysogenic cycle). In the lysogenic cycle, the phage-derived genome is termed a prophage. The proteins expressed by the newly formed genome results in the modification of the host cell metabolism. The lysogenic cycle has medical importance since the toxic effect of some bacterial infections is caused by prophages. For example, diphtheria toxin (produced by *Corynebacterium diphtheriae*) and Shiga Toxin (produced by *E. coli* and *Shigella flexneri*) are prophage induced [7,8].

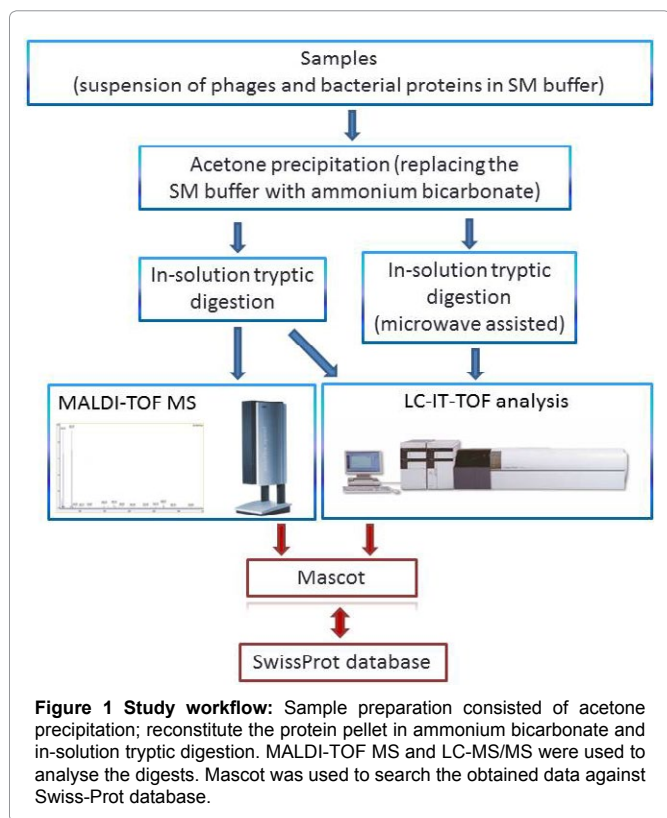
Another medical importance of bacteriophage is in their potential use for treating bacterial infections, emphasised by the emergence of antibiotic resistance which represents a critical problem in today's medicine. Administration of bacteriophages for treating bacterial infections was considered even before the discovery of antibiotics. Although this approach is declining in the United States and Western Europe once the use of antibiotics became widespread, phages continued to be used in Eastern Europe where several studies were published on their potential use [9-11].

Until now, mass spectrometry was employed in several studies for characterisation of the proteome of several species of phages [12-18]. However, in these studies, complex sample techniques were employed. Thus, either gradient centrifugation [13,14] or polyacrylamide gel electrophoresis [12,15,18] were used for separating and concentrating the viral proteins before mass spectrometry analysis.

The aim of the present study was to develop a rapid and reliable mass spectrometry-based proteomic method for identification of *Escherichia coli* phages. The possibility of applying the methodology for detecting other phages was also taken into consideration. The mass spectrometry approach can eliminate the need of dedicated materials such as primers and probes used in genomic approaches. Moreover, the proteomic approach can achieve a level of multiplexing where several species of phages can be targeted without changing the method parameters and without using specialised materials. The approach took into consideration the restricted number and low concentration of phage proteins when compared with the host bacteria. Also, the complexity of the sample preparation process was taken into account. This was simplified and complex procedures such as density gradient centrifugation, gel electrophoresis and size exclusion filtration were avoided since they are labour intensive and time consuming. Therefore, the infected *E. coli* cultures were directly subjected to in-solution tryptic digestion. The resulting peptides were analysed using

\*Corresponding author: Serafim Vlad, Department of Natural Sciences, Middlesex University, The Burroughs, London NW4 4BT, United Kingdom, Tel: 0044 2084114586, 0044 7876383303; E-mail: V.Serafim@mdx.ac.uk

Received: January 23, 2017 Accepted: February 21, 2017 Published: February 28, 2017



MALDI-TOF MS and LC-MS/MS. The study workflow is illustrated in Figure 1.

The possibility of identifying prophage-induced proteins was also investigated. Thus, the toxic potential of *E. coli* and *Shigella flexneri* [8] could be evaluated and the potential Shiga Toxin-producing strains could be identified in a very short time and with relatively low costs. This technique could be used as an alternative to the DNA detection tests [19] where a complex sample preparation and multiple DNA probes are required for detection of different phage species.

## Methodology

### Materials

Sodium chloride, magnesium sulphate, Tris-hydrochloride, gelatin, acetone, dithiothreitol, iodoacetamide, trypsin from porcine pancreas of proteomics grade, formic acid, trifluoroacetic acid, acetonitrile, HPLC-grade water and  $\alpha$ -cyano-4-hydroxycinnamic acid were purchased from Sigma-Aldrich, UK. C18 ZipTip were purchased from Merck Millipore (Hertfordshire, UK).

### Safety considerations

All hazardous substances were handled using appropriate protective gloves, clothing and eye protection. Formic acid, trifluoroacetic acid, acetone and acetonitrile here handled inside the fume cupboard.

### Preparation of phage suspension

SM buffer was prepared using the following recipe: 100 mM NaCl, 10 mM  $MgSO_4$ , 50 mM Tris-HCl (pH7.5), 0.01% (w/v) gelatin.

Phages, isolated from sewage effluent and suspensions of human

faeces (unpublished work), were diluted in SM buffer and added to 200  $\mu$ L of overnight cultures of host bacteria (*E. coli* strain 1025). The mixture was then added to 3 mL aliquots of 'top agar' (Nutrient broth + 0.7% agar) and plated on 90 mm Nutrient agar plates. Following overnight incubation at 37°C, isolated plaques were picked into SM buffer and re-plated with host bacteria as before. On this occasion, plates exhibiting around 90-95% confluent lysis were selected. Five mL of SM buffer was added to the surface of these plates and left at 4°C overnight for phage to soak into the buffer. These phages 'soaks' were removed from the agar surface using a pipette clarified using centrifugation at 10,000 g for 10 min and stored at 4°C until further processing.

### Protein extraction

Phage suspensions in SM buffer were treated with ice-cold acetone 1:5 suspension : acetone (1:5, v/v). The samples underwent centrifugation at 13,000 g for 5 min. The supernatant was discarded and the pellet was allowed to dry. The pellet was reconstituted in 50 mM ammonium bicarbonate at the initial sample volume. The mixture was sonicated using Soniprep 150 Plus (MSE, UK) for 10 s with the amplitude set at 13.

### In-solution tryptic digestion

An aliquot of phage suspension (50  $\mu$ L) was treated with 3  $\mu$ L of 100 mM dithiothreitol in 50 mM ammonium bicarbonate prior to heating at 95°C for 5 min. The samples were allowed to cool to room temperature and then 6  $\mu$ L of 100 mM iodoacetamide in 50 mM ammonium bicarbonate was added. The mixture was incubated in the dark, at room temperature for 20 min. A 2  $\mu$ L aliquot of trypsin (0.1  $\mu$ g/ $\mu$ L dissolved in 50 mM ammonium bicarbonate) was added and tube was incubated at 37°C for 3 h. A further 2  $\mu$ L of trypsin was added to the sample tube and the mixture was incubated at 37°C for a further 2 h.

For microwave-assisted digestion, 2  $\mu$ L of trypsin (0.1  $\mu$ g/ $\mu$ L) was added to each sample (50  $\mu$ L in volume) and the tubes were placed in a plastic beaker containing 250 mL of tap water. The beaker with samples was then placed in a domestic microwave oven which was set at the highest setting (700 W) and operated for 2 min. The tubes were removed and a further 2  $\mu$ L of trypsin was added. The samples were microwaved using the same settings as before. Following tryptic digestion the samples were allowed to cool to room temperature.

### MALDI-TOF/MS analysis of tryptic digests

Prior to spotting samples onto the MALDI target plate, the digests were processed using ZipTip C18 pipette tips following the manufacturer's protocol. Subsequently, an aliquot (1  $\mu$ L) of digest was spotted on a 96 wells polished steel MALDI-TOF plate (Bruker Daltonics, UK) After drying 1  $\mu$ L of 5 mg/mL of  $\alpha$ -cyano-4-hydroxycinnamic acid (HCCA) in a mixture of acetonitrile, water and trifluoroacetic acid (50:47.5:2.5%,v/v) was applied. Once dried the samples were analysed using a Bruker Autoflex III (Bruker Daltonics, Coventry, UK) MALDI-TOF mass spectrometer. For peptide the fingerprinting was acquired in positive ion reflectron mode. The  $m/z$  range was set to 700-3,500 and acceleration voltage to 1958 V. The instrument was calibrated using peptide calibration standard II (Bruker Daltonics, UK) the data was acquired in positive ion reflectron mode. The mass range was set to 680-2,256 and the acceleration voltage to 1810 V.

The mass spectra were processed using the Bruker FlexAnalysis

(version 3.4) software. The obtained peptide mass fingerprints were searched against all entries in Swiss-Prot database (2016\_2) using the Mascot (Matrix Science, UK). The search parameters were set as follows: trypsin as the cleavage enzyme, carbamidomethylation of cysteine (variable modification) and oxidation of methionine (constant modification). The mass tolerance was set to 250 ppm and two miss cleavages were allowed.

### LC-MS/MS analysis of tryptic digests (full ion scan)

All LC-MS analyses were carried out using a Shimadzu Prominence HPLC system hyphenated to an electrospray ionisation hybrid ion-trap time-of-flight (IT-TOF) mass spectrometer (Shimadzu, UK). For chromatographic separation a 150 × 2.1 mm, 2.7 μm C18 column (Ascentis Express C18 – Sigma-Aldrich) was used. LabSolutions® software (version 3.50.348, Shimadzu, UK) was used for controlling the instrument and for acquiring the data. Separation of peptides was performed using a linear gradient elution profile composed of 'A'-0.1% formic acid in water and 'B'-0.1% formic acid in acetonitrile at a flow rate of 0.21 mL / min. The elution profile started at 0% B then increased to 40% B over 50 min then to 90% 'B' over 1 min, maintained at this level for 3 min and then returning to 0% B in 1 min and kept at this composition for 15 min to equilibrate the column. A volume of 10 μL was injected per run and the column oven temperature was set at 40°C. Samples were kept in the autosampler tray set to 4°C. Mass spectrometry analysis was performed in MS/MS mode using positive ions electrospray. The precursors' acquisition range was set from 400-1,800 *m/z* while the fragments acquisition range was set from 200-1,500 *m/z*. For both precursors and fragments the ion accumulation time was set to 30 msec. The detector voltage was set to 2 kV and CID energy was set at 50%. The other instrument conditions were set as follows: nebulising gas flow 1.5 L/min, CDL temperature 200°C, heat block temperature 200°C, interface voltage 4.5 V, detector voltage 2 kV. The data acquisition was performed in a 37.5 min interval.

### LC-MS/MS single ion monitoring method for identifying the phage-induced ea10 protein

The targeted peptide had a unique sequence and a theoretical *m/z* of 1114.5327 (6% of the total protein sequence). In the full ion scan, it was detected as the double charged ion at 558.27 *m/z*. Thus, the precursor selection was set between 558.00 and 558.47 *m/z*. Precursor charge state was limited at 2+. Fragments selection range was set at 200-1000 *m/z* with a tolerance of 0.05 Da. Fragments ion accumulation was increased at 50 msec. The rest of the parameters were as previously described. The rest of parameters were left as described before.

### LC-MS/MS data processing

MS/MS data were extracted from the resulting instrument files using Mascot Distiller software (version 2.5.1.0, Matrix Science, London, UK). The MS/MS ion list was searched using Mascot search engine against all entries in Swiss-Prot database (2016\_2).

For database search the following parameters were used: two missed cleavages, carbamidomethylation of cysteine (as fixed modification) and oxidation of methionine (as variable modification). For full ion scan, the tolerance for precursor peptides was set to 15 ppm and for fragments to 0.3 Da. Peptide charges used for peak picking was +2, +3 and +4. For single ion monitoring, the tolerance the parent ion was set at 5 ppm and for fragments at 0.05 Da.

## Results and Discussion

Following MALDI-TOF MS analysis of the tryptic digests, two bacteriophage species were identified: Enterobacteria phages Lambda and T4. The major capsid protein was identified for both species (Table 1).

Following LC-MS/MS analysis, five phages species were identified based on their protein composition. These were Enterobacteria phages P2, T4, T5, T7 and Lambda. The proteins identified for these organisms are listed in Table 2 together with the number of corresponding identified peptides and their individual Mascot scores. For all proteins, the identification was based on fragmentation patterns of unique peptides. Only ions which exhibited identity or extensive homology (the ion cut-off score was adjusted according to Mascot calculation) were selected. Viral proteins specific only to a single bacteriophage species were identified in each sample. For each bacteriophage species, the number of identified protein varied from one for P2 phage to twelve for T5 phage.

In-solution digestion was preferred over in-gel digestion since it is significantly less labour-intensive and takes less time to perform. The tryptic digests were separated using liquid chromatography using a fused-core C18 column which, together with the mass analyser, provided an excellent resolution to identify all the bacteriophage species in a complex mixture of bacterial host proteins. Furthermore, the in-solution trypsination was accelerated significantly by using microwave assisted digestion.

More bacteriophage proteins (Table 2) and higher protein scores were obtained using 5 h digestion protocol than the microwave assisted method. Although less confident, the microwave-assisted digestion provided identification of all bacteriophage species used in this study.

MALDI-TOF MS proved to be less successful in identifying the bacteriophage digests. The intensities of the parent ions were very low so they could not be analysed in MS/MS mode using laser-induced fragmentation. The reason for this might have been the low concentration of the phage proteins. All this considered, the LC-IT-TOF proved to be more suitable for proteomics-based identification of bacteriophage. The chromatographic separation combined with ion accumulation provided greater selectivity and sensitivity that allowed the identification of viral proteins in an overwhelming mixture of bacterial proteins.

The LC-MS/MS full scan protein report also revealed the Lambda prophage-derived protein ea10. However, due to the high number

Table 1: Bacteriophage proteins identified using MALDI-TOF MS.

Species	Identified protein	Protein MW (Da)	No. of matched masses	Protein coverage	Mascot score
Enterobacteria phage lambda	Major capsid protein	38221	9	34%	114
Enterobacteria phage T4	major capsid protein	56043	9	29%	112

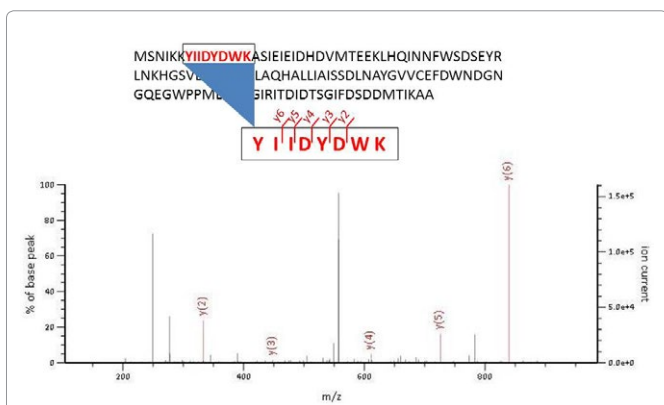
The peptide mass fingerprints were obtained in reflectron mode using HCCA as matrix. The mass range was 700-3,500 *m/z*. The data was searched against SwissProt database using Mascot.

**Table 2:** Bacteriophage proteins identified using LC-IT-TOF in full MS/MS mode (peptide fragmentation fingerprint).

Species	Protein	MW (Da)	5-hours digestion			MW-assisted digestion		
			Number of peptide identified	Protein score	Protein coverage	Number of peptides identified	Protein score	Protein coverage
Enterobacteria phage T4	Major capsid protein	56,043	8	476	21%	6	273	15%
	Tail fiber assembly helper protein	8,726	4	165	55%	3	80	33%
	Fibritin	51,840	3	150	12%	1	59	5%
	Capsid vertex protein gp2447149	46,994	2	56	5%	ND*	ND*	ND*
	Baseplate wedge protein gp8	38,268	1	43	3%	ND*	ND*	ND*
	Uncharacterized 10.2 kDa protein	10,375	1	40	10%	ND*	ND*	ND*
	Prohead assembly protein gp68	15,864	1	39	7%	ND*	ND*	ND*
Tail tube protein gp19	18,507	1	39	11%	ND*	ND*	ND*	
Enterobacteria phage P2	Capsid protein	40,335	3	117	15%	1	77	4%
Enterobacteria phage T7	Major capsid protein	36,637	3	149	9%	3	136	9%
	Portal protein	59,257	1	64	2%	ND*	ND*	ND*
	DNA ligase	41,449	1	54	3%	ND*	ND*	ND*
	Tail fiber protein	61,705	1	45	1%	ND*	ND*	ND*
	Single-stranded DNA-binding protein gp2.5	25,849	1	57	10%	ND*	ND*	ND*
	Capsid assembly scaffolding protein	33,877	1	52	3%	1	69	3%
Enterobacteria phage lambda	Major capsid protein	38,221	20	686	55%	7	241	30%
	DNA-packaging protein	14,299	4	117	45%	ND*	ND*	ND*
	Tail assembly protein GT	31,151	1	99	8%	1	40	10%
	Tail tube protein	25,794	1	81	6%	ND*	ND*	ND*
	Capsid assembly protease	46,139	1	45	2%	ND*	ND*	ND*
	Serine/threonine-protein phosphatase	25,431	1	44	5%	ND*	ND*	ND*
Enterobacteria phage T5	Capsid decoration protein	11,565	ND*	ND*	ND*	2	97	41%
	Probable ssDNA-binding protein	29,006	5	161	27%	ND*	ND*	ND*
	L-alanyl-D-glutamate peptidase	15,255	3	141	33%	1	61	11%
	Tail tube protein	50,439	3	126	9%	2	92	9%
	Prohead protease	23,592	2	73	12%	ND*	ND*	ND*
	Probable terminase	17,987	1	68	8%	ND*	ND*	ND*
	Deoxyuridine 5'-triphosphate nucleotidohydrolase	16,307	3	63	23%	ND*	ND*	ND*
	Portal protein	45,287	1	49	1%	1	38	6%
	Probable thymidylate synthase	32,070	2	45	8%	ND*	ND*	ND*
	Deoxynucleoside-5'-monophosphate kinase	28,854	1	44	4%	ND*	ND*	ND*
	Major capsid protein	50,853	1	42	2%	1	39	3%
Probable tape measure protein	131,415	1	36	1%	ND*	ND*	ND*	
Head completion protein	19,255	ND*	ND*	ND*	1	59	5%	

The chromatographic was performed using a C18 column with binary gradient. Mass spectrometry was performed positive MS/MS mode with an acquisition range of 400 – 1800 *m/z* for precursors and 200 – 1500 *m/z* for products. Mascot Distiler was used for processing the data and the search was performed against SwissProt database.

ND\* - not detected



**Figure 2: LC-MS/MS data for the peptide selected for identification the Lambda prophage derived protein ea10.**

Mass spectrometry was performed positive MS/MS mode with an acquisition range of 558.00 – 558.47 *m/z* for precursor and 200 – 1000 *m/z* for products. Mascot Distiller was used for processing the data and the search was performed against Swiss rot database. The protein amino acid sequence is provided above the spectrum and the selected peptide is marked in red. The product ions are also marked corresponding to the spectrum below.

of values searched and the database complexity, the scores obtained placed the identification score below the homology threshold. Therefore, in order to develop a specific identification method, a single ion monitoring approach was used in order to isolate and fragment a unique peptide originating in ea10 protein. The results obtained showed a significant score (37 when scores >30 indicate identity or extensive homology) which provided a confident identification for the prophage peptide (Figure 2).

## Conclusions

The results showed that presence of bacteriophage-encoded proteins originating from both lytic and lysogenic infections. For the prophage-induced protein, present at a low concentration, a single ion monitoring method can be used. This emphasises the need to carry out further LC-MS/MS of analyses of proteins extracts from bacteria which contain phages in lysogenic stage. Also, higher resolution LC-MS instruments can be used for increasing the selectivity and sensitivity. Thus, a method for detection of prophages based on protein biomarker could be developed as an alternative to the genomic detection. The proteomic based method could be cheaper, faster, and have a significant level of multiplexing since it can identify several phage species using the same parameters.

## References

1. Shah HN, Gharbia SE (2010) Mass Spectrometry for Microbial Proteomics. John Wiley and Sons Ltd, London, United Kingdom.
2. Maxwell KL, Frappier L (2007) Viral Proteomics. *Microbiol Mol Biol Rev* 71: 398-411.
3. Trauger SA, Junker T, Siuzdak G (2013) Investigating Viral Proteins and Intact Viruses with Mass Spectrometry. *Top Cur Chem* 225: 265-282.
4. Murray PR (2012) What Is New in Clinical Microbiology-Microbial Identification by MALDI-TOF Mass Spectrometry. *J Mol Diagn* 14: 419-423.
5. Goering R, Dockrell H, Zuckerman M, Roitt I, Chiodini PL (2013) *Mims' Medical Microbiology*, (5edn) Elsevier. United Kingdom.
6. Mc Grath S, van Sinderen D (2007) *Bacteriophage: Genetics and Molecular Biology*. Caister Academic Press. United Kingdom.
7. Black JG (2013) *Microbiology* (8th edtn), John Wiley & Sons Ltd. London, United Kingdom.


8. Friedman DI, Court DL (2001) Bacteriophage lambda: alive and well and still doing its thing. *Curr Opin Microbiol* 4: 201-207.
9. Bogovazova GG, Voroshilova NN, Bondarenko VM, Gorbatkova GA, Afanas'eva EV, et al. (1992) Immunobiological properties and therapeutic effectiveness of preparations from Klebsiella bacteriophages. *Zh Mikrobiol Epidemiol Immunobio* 3: 30-33.
10. Carlton RM (1999) Phage therapy: past history and future prospects. *Arch Immunol Ther Exp (Warsz)* 5: 267-274.
11. Sulakvelidze A, Alavidze Z, Morris JG (2001) Bacteriophage Therapy. *Antimicrob Agen Chem* 45: 649-659.
12. Borriss M, Lombardot T, Glockner FO, Oliver F, Becher D, et al. (2007) Genome and proteome characterization of the psychrophilic Flavobacterium bacteriophage 11b. *Extremophiles* 11: 95-104.
13. Chang JR, Poliakov A, Prevelige PE, Mobley JA, Dokland T (2008) Incorporation of scaffolding protein gpO in bacteriophages P2 and P4. *Virology* 370: 352-361.
14. Duplessis M, Le'vesque SM, Moineau S (2006) Characterization of *Streptococcus thermophilus* Host Range Phage Mutants. *Appl Environ Microbiol* 72 : 3036-3041.
15. Eyer L, Pantůček R, Zdráhal Z, Konečná H, Kaspárek P, et al. (2007) Structural protein analysis of the polyvalent staphylococcal bacteriophage 812. *Proteomics* 7: 64-72.
16. Lavigne R, Noben JP, Hertveldt k, Ceyskens PJ, Briers Y, et al. (2006) The structural proteome of *Pseudomonas aeruginosa* bacteriophage wKMV. *Microbiol* 152: 529-534.
17. Lavigne R, Ceyskens PJ, Robben J (2009) Phage proteomics: applications of mass spectrometry. *Methods Mol Biol* 502: 239-51.
18. Ahmad AA, Ogawa M, Kawasaki T, Fujie M, Yamada T (2014) Characterization of Bacteriophages Cp1 and Cp2, the Strain-Typing Agents for *Xanthomonas axonopodis* pv. Citri. *Appl Environ Microbiol* 80: 77-85.
19. Gabig-Ciminska M, Los M, Holmgren A, Albers J, Czyz A, et al. (2004) Detection of bacteriophage infection and prophage induction in bacterial cultures by means of electric DNA chips. *Anal Biochem* 324: 84-91.

## Author Affiliation

Top

Department of Natural Sciences, Middlesex University, The Burroughs, London NW4 4BT, UK

## Submit your next manuscript and get advantages of SciTechnol submissions

- ❖ 80 Journals
- ❖ 21 Day rapid review process
- ❖ 3000 Editorial team
- ❖ 5 Million readers
- ❖ More than 5000 
- ❖ Publication immediately after acceptance
- ❖ Quality and quick review processing through Editorial Manager System

Submit your next manuscript at • [www.scitechnol.com/submission](http://www.scitechnol.com/submission)