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Isolation of a New *Neisseria* Phage from the Oral Cavity of Healthy Humans

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Abstract

The incidences of antibacterial drug resistant pathogens are rising, which necessitates the need for alternative therapies. As a result, research in the field of antibiotic drug development utilising bacteriophage is gaining momentum. The aim of this study is to provide proof of concept that phages can be isolated from healthy human individuals and that such phages may play a significant role in the maintenance of oral health. The results of sequence analyses using the available databases, confirmed the presence and identification of different phage proteins, which have been inserted into a bacterial host identified as *Neisseria meningitidis*. The results in this study warrant further investigation into the efficacy and suitability of the isolated phage as potential control for oral infections and possibly the development of a selective phage therapy treatment for *Neisseria*.

Keywords: *Neisseria*; Bacteriophage; DNA sequence

Introduction

There is no effective or definitive therapy to treat periodontal disease, which is a wide set of pathological alterations that occur in the human oral cavity to which different bacterial groups have been suggested to be the causative microorganism [1]. These bacterial groups are usually embedded in dental plaques and represent a complex microbial community, widely known to be the precursor for many infections including gingival [2]. While, gram negative anaerobic bacteria species have predominantly been isolated from infected individuals, they have also been isolated from healthy individuals [3]. The plaque biofilm is stabilised and protected by a bacterially-produced exopolysaccharide matrix [4]. Therefore, specific disruption of the bacterial polysaccharide may provide a new therapy to treat not only periodontal causing bacteria, but also to treat a wider group of antibiotic resistant bacterial strains.

Bacteriophages are bacteria-specific viruses that infect, and in the case of obligate lytic phages, destroy their host bacteria [5]. Since their discovery in the early nineteenth century, bacteriophages were clinically used as antibacterial agents until the discovery of penicillin, after which the use of bacteriophage as antibacterial agents was replaced by the new therapies [5]. Bacteriophages have the potential to regulate the oral microflora by i) lysing sensitive cells, ii) selecting mutants (which may have altered characteristics) and iii) by releasing bacterial components with pro-inflammatory activity [6]. Intriguingly, many examples of phage therapies at the clinical and commercial levels have been documented [7,8], including their use in dentistry where several bacteriophages that infect diverse oral bacteria have been isolated from saliva and dental plaques [9-11].

This study describes the isolation of a new phage from *Neisseria meningitides* strain (WUE2594), which were attained from 3 healthy human plaque samples. The study provides proof of concept that phages are present in dental plaques of healthy individuals and that they could potentially provide a selective antibacterial activity against their host species *Neisseria*.

2. Detecting, Purification and DNA Sequencing of a New Bacteriophage

LB and BHIB agar were used as a bottom agar, while 0.35% agarose LB and BHIB were used as the soft top agar, which were supplemented with horse blood at 2%, 3%, 5% v/v, to ensure growth for bacteria that grew better in the presence of blood. For infection, 100 µl of filtered sample of was added to 300 µl of the host cell culture that had been grown overnight. The viral particles were allowed to adsorb into the host cells for 15 min at room temperature, then the infected cells were added to 3 ml of the molten soft top agar in universal tubes and mixed well before being poured onto the bottom agar. This was left to set for a few minutes; the plates were then inverted and incubated at 37°C. After 24 to 48 h, they were checked for the appearance of plaques.

Completely lysed soft top agars were collected and added to 40 ml of SM buffer (Krackeler Scientific, US) and then incubated overnight at 10°C to allow the virus particles to diffuse from the soft top agar into the SM buffer. The sample was then centrifuged at 250× g for 25 min, and then the supernatant was filtered with a 0.45 µm and then a 0.22 µm Millipore filter to ensure the removal of agar and cell debris. The viral particles were mixed with 1/8 volume Polyethylene glycol (PEG) 6000 solution (2.5 M NaCl, 20% (w/v) PEG 6000) and incubated on ice for 30 min. Samples were then centrifuged at 16000× g for 10 min and the virus pellet was re-suspended in 0.5 ml of 10 mM Tris pH 7.5, 10 mM $MgCl_2$, 100 mM NaCl. Free nucleic acids were digested by adding 10 U of DNase and 10 µg/ml RNase A and incubating for 30 min at 37°C. Nucleic acids were then extracted using an equal volume of phenol: chloroform.

Extracted viral genomes were cut using restriction endonuclease (New England Biolabs). DNA fragments of 0.5 to 2 kb in size were ligated to the pGEM-T Easy® Promega vector, and introduced into competent *E. coli* JM109. The complete nucleotide sequence

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was analysed using Nucleotide Basic Local Alignment Search Tool (BLASTN) to search for highly similar sequence alignments within the nucleotide collection database. The complete contig was entered into MacVector 12.5 sequence analysis software and open reading frames (ORFs) 150 nucleotides or greater ≥ 50 amino acids) were identified. The ORFs were translated using MacVector 12.5 and the protein sequences were entered into Protein BLAST (BLASTP) and used to search for non-redundant protein sequence alignments.

While we have not attempted to characterise these viral proteins, our preliminary database search showed that the majority of these sequences have high homology to viral (phage) proteins (Table 1). These viral proteins were identified by setting up the selection search to the minimum size of the ORF's to be more than 50 amino acids in length. Generally, the majority of the identified phage related proteins seemed to be associated with an insertion into the *Neisseria* genome. A significant number of the identified phage proteins have strong association with *Neisseria meningitides*, which might suggest that the identified virus might be a specific prophage for the bacteria *Neisseria meningitidis*. However, based on the fact that almost all of the rest of the identified proteins were associated with insertion into different *Neisseria* genome, we can safely speculate that the identified virus is a specific phage for the *Neisseria* genus.

Furthermore, the preliminary sequence analyses have revealed a number of interesting observations including findings of putative bacterial promoter sequences, which were identified at -35 (TTGACA) and -10 (TATAAT). Intriguingly, these sequences were identified for the vast majority (79%, 19/24) of the identified phage related genes (Figure 1). However, it is rather difficult to accurately identify the exact promoter sequence for the identified phage proteins without experimental validation. Hence, further analyses and characterisation of the identified phages are being undertaken at a high scale by our research group.

Discussion and Conclusion

There is an ever constant increase in the reported cases of multiple antibiotic-resistant pathogenic bacteria [12], which has prompted many researchers to revisit an older antibacterial therapy that utilises bacteriophages. Multi drug resistant bacterial pathogens pose a major threat to human health as well as to the long term efficacy of commonly used antibiotics [13]. The last few years have seen a significant increase in the number of new bacteriophage research programs, encompassing different delivery routes, the most popular being oral and parenteral [5,11]. Research that focuses on the oral route of 'anti-bacterial' therapy revolves around the scenario of the potential isolation of specific bacteriophages from the human oral cavity, and investigating the possibility of utilising these phages as potential antibacterial agents. Bacteriophages isolated from the human oral cavity will more likely be useful in the development of antibacterial therapies for antibiotics resistant oral pathogens. Also, most of the published reports on human oral lytic phage isolation have encountered and/or reported the formation of lysis zones [2].

Bachrach *et al.* (2003) [14] reported the isolation of a lytic bacteriophage which they speculate contributes to the ecosystem of the human oral cavity and also possibly to overall human health since the phage is ubiquitously associated with its bacterium host. Oral pathogens are therefore noted to be found both in healthy and diseased individuals [3]. Thus, the presence of phages in healthy individuals may, although does not necessarily prove, the theory that they, phages, somehow contribute to the overall health of the oral flora. While a number of studies who reported the isolation of bacteriophages from healthy human individuals indicated somewhat the likelihood of phages contributing on maintaining the oral flora [1,11,15], many others have argued otherwise [16,17].

This study provides proof of concept that bacteriophages may be isolated from healthy human individuals and that this fact (isolation from healthy individuals), prompted us to speculate that the isolated phage is likely to play a role in the maintenance of the oral flora. This warrants further investigations into the promising utility of bacteriophages therapy as an antibacterial modality. Furthermore, the identified bacteriophage could be utilised or further developed into making specific antibiotic treatments that could potentially target its host, *Neisseria meningitidis*, or the *Neisseria* species in general. Currently, we are further validating and characterising this isolated phage as well as determining the biological role that these phages might play in health and disease- which we believe can significantly improve the development of specific bacteriophage therapy.

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Conflict of Interest

Non declared.

Description	Max score	Total score	Query coverage	E value	Max ident
prophage pi1 protein 11, recombinase [Neisseria subflava NJ9703] >gb|EFC51410.1| prophage pi1 protein 11, recombinase [Neisseria subflava NJ9703]	468	468	99%	7e-165	99%
COG3723: Recombinational DNA repair protein (RecE pathway) [Actinobacilluspleuropneumoniaeserovar 1 str. 4074]	264	264	99%	1e-84	59%
recombinational DNA repair protein [Acinetobacterjunii SH205] >gb|EEY94076.1| recombinational DNA repair protein [Acinetobacterjunii SH205]	260	260	98%	4e-83	57%
putative recombinational DNA repair protein, RecE pathway [Mannheimiahaemolytica serotype A2 str. OVINE] >gb|EEY09846.1| putative recombinational DNA repair protein, RecE pathway [Mannheimiahaemolytica serotype A2 str. OVINE]	259	259	99%	6e-83	59%
recombinational DNA repair protein, RecE pathway [Haemophilusaegyptius ATCC 11116] >gb|EGF19332.1| recombinational DNA repair protein, RecE pathway [Haemophilusaegyptius ATCC 11116]	253	253	99%	3e-80	58%

Table 1: Gene 6- top 5 alignments

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Query          1    MAATLNLPINNSLGFAYIVPFQ------NRK--------EN-------V-------T---  29
ZP_05985602    64   MAATLNLPINNSLGFAYIVPFQ------NRK--------EN-------V-------T---  92
ZP_00135136    72   MAATLNLPLQNGLGFAYIVPYQ------NRK--------EK-------K-------T---  100
ZP_06066245    70   MAATLNLPINNNLGFAYIVPFK------NNK--------EN-------K-------I---  98
ZP_05992197    69   MAATLNLALQNGLGFAYIVPYQ------NRK--------EK-------K-------T---  97
ZP_08250835    68   MAATLNLPLQNGLGFAYIVPFR------NNK--------EK-------K-------T---  96

Query          30   --EAQFQLGYKGFIQLAQRSGQFKRINACPVYD-TD-V--------E-------------  64
ZP_05985602    93   --EAQFQLGYKGFIQLAQRSGQFKRINACPVYD-TD-A--------E-------------  127
ZP_00135136    101  --EAQFQLGYKGLIQLAQRSGQFKRLVAVPVYE-KQLI--------A-------------  136
ZP_06066245    99   --EAQFQLGYKGYIQLAQRSGQFSRIAATPVYD-GQLI--------S-------------  134
ZP_05992197    98   --EAQFQLGYKGLIQLAQRSGQFKRLVAVPVYE-KQ-LL-------A-------------  133
ZP_08250835    97   --EAQFQIGYKGFIQLAQRSGQFKRLVALPVYK-KQLI--------K-------------  132

Query          65   -E----DVYQRLT-------S--L--------I---------P----R----K--P--S-G  81
ZP_05985602    128  -E----DVYQRLT-------S--L--------I---------P----R----K--P--S-G  144
ZP_00135136    137  -E----DPINGFE-------F--D--------W---------K----Q----K--P--A-K  153
ZP_06066245    135  -E----NPLLGYE-------F--D--------W---------S----V----K--P--N-G  151
ZP_05992197    134  -E----DPINGYE-------F--D--------W---------K----Q----K--P--A-K  150
ZP_08250835    133  -K----DFINGFE-------F--D--------W---------E----Q----E--PEQN-E  151

Query          82   ----Q----I-IGYIAYFQLLNGYEANLTMTMEELEAH--AKRYSQT-----Y-------  118
ZP_05985602    145  ----Q----I-IGYIAYFQLLNGYEANLTMTMEELEAH--AKRYSQT-----Y-------  181
ZP_00135136    154  DE--K----P-IGYYAYFKLINEFTAELYMSTQDVYDH--AARYSQT-----Y-------  192
ZP_06066245    152  ----N----P-IGYVAFFKLINGFTAELYMSKEEVMKH--ANKYSQT-----A-------  188
ZP_05992197    151  DE--K----P-IGYYAYFKLINEFTAEIYMTTQEVHDH--ANRYSQT-----Y-------  189
ZP_08250835    152  ----N----P-IGYYAYFKLVNDFSAELYMSHDDIVKH--AQRYSQT-----F-------  188

Query          119  ---K----------R----------G-------F--G------V----------------  124
ZP_05985602    182  ---K----------R----------G-------F--G------V----------------  187
ZP_00135136    193  ---K----------K----------G-------Y--G------V----------------  198
ZP_06066245    189  ---K----------K----------G-------Y--G------V----------------  194
ZP_05992197    190  ---R----------K----------G-------Y--G------V----------------  195
ZP_08250835    189  ---K----------K----------G-------Y--G------V----------------  194

Query          125  -----W--A-DN--FEAMAKKTVIKLLLSQQAPLSIEMQ------KAVLAD-----Q---  160
ZP_05985602    188  -----W--A-DN--FEAMAKKTVIKLLLSQQAPLSIEMQ------KAVLAD-----Q---  223
ZP_00135136    199  -----W--H-DN--FEAMAIKTVLKLLLSKQAPLSVEMQ------QAVLAD-----Q---  234
ZP_06066245    195  -----W--K-DQ--FEAMALKTVLKLLLSKQAPLSIDMQ------KAQMAD-----Q---  230
ZP_05992197    196  -----W--H-DN--FEAMALKTVMKLLLSKQAPLSMEMQ------QAVLAD-----Q---  231
ZP_08250835    195  -----W--H-DN--FEAMALKTVTKLLLSKQAPLSVEMQ------QAVLAD-----Q---  230

Query          161  ------A-----I-V------K----D--V----E----A-------E-----E------  170
ZP_05985602    224  ------A-----I-V------K----D--V----E----A-------E-----E------  233
ZP_00135136    235  ------A-----V-V------K----D--V----E----N-------A-----E------  244
ZP_06066245    231  ------A-----I-I------R----D--V----D----K-------D-----E------  240
ZP_05992197    232  ------A-----V-V------K----D--V----E----N-------A-----E------  241
ZP_08250835    231  ------A-----V-V------K----D--V----E----N-------Q-----E------  240

Query          171  F-----------EYIDNQP--M-PA--ETP--KMA-VSDE-M--FEQLKENISTGDIDIQ  208
ZP_05985602    234  F-----------EYIDNQP--M-PA--ETP--KMA-VSDE-M--FEQLKENISTGDIDIQ  271
ZP_00135136    245  F-----------SYPDNEV--Q-EA--EFT--ELK-VNDD-V--FEKCKQNIISGDATLQ  282
ZP_06066245    241  F-----------DYIDHQE--S-IADLEAP--KPT-LNDD-E--FNAALEQLNVGAIDKA  280
ZP_05992197    242  F-----------AYPDNEI--Q-EA--EFT--ELK-VSDE-V--FEKCKQTIINKESTLQ  279
ZP_08250835    241  F-----------NYTDN------IQ--EAE--FLA-VVDEAT--FEQCKQSIANGETTLQ  276

Query          209  TVL--DS--YDLSEEQKAELDKL  227
ZP_05985602    272  TVL--NS--YDLSEEQKAELDKL  290
ZP_00135136    283  DLC--DNG-YEFSKEQYAILEEI  302
ZP_06066245    281  YIL--DG--YSLTDAQRVAVE    297
ZP_05992197    280  DMC--DNG-YEFNKEQYAELEKL  299
ZP_08250835    277  ELC--DSGAYEFSQEQIAELEAI  297
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Figure 1: Identified putative bacterial phage related genes.

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