Salmonella Typhimurium-specific bacteriophage \( \Phi SH19 \) and the origins of species specificity in the Vi01-like phage family

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Abstract

Background: Whole genome sequencing of bacteriophages suitable for biocontrol of pathogens in food products is a pre-requisite to any phage-based intervention procedure. Trials involving the biosanitization of Salmonella Typhimurium in the pig production environment identified one such candidate, \( \Phi SH19 \).

Results: This phage was sequenced and analysis of its 157,785 bp circular dsDNA genome revealed a number of interesting features. \( \Phi SH19 \) constitutes another member of the recently-proposed Myoviridae Vi01-like family of phages, containing \( S. Typhi \)-specific Vi01 and \( Shigella \)-specific SboM-AG3. At the nucleotide level \( \Phi SH19 \) is highly similar to phage Vi01 (80-98% pairwise identity over the length of the genome), with the major differences lying in the region associated with host-range determination. Analyses of the proteins encoded within this region by \( \Phi SH19 \) revealed a cluster of three putative tail spikes. Of the three tail spikes, two have protein domains associated with the pectate lyase family of proteins (Tsp2) and P22 tail spike family (Tsp3) with the prospect that these enable \( Salmonella \) O antigen degradation. Tail spike proteins of Vi01 and SboM-AG3 are predicted to contain conserved right-handed parallel \( \beta \)-helical structures but the internal protein domains are varied allowing different host specificities.

Conclusions: The addition or exchange of tail spike protein modules is a major contributor to host range determination in the Vi01-like phage family.

Keywords: Phage biocontrol, biosanitization, bacteriophage genomics, Salmonella Typhimurium, Myoviridae, P22-like tail spike, pectate lyase tail spike domain, lipopolysaccharide

Background

The use of virulent bacteriophages (phages) as biological control (biocontrol) agents against bacterial pathogens is an expanding field of research aimed at producing sustainable solutions for the control of these pathogens, and to circumvent problems such as those associated with the development of multidrug-resistant bacteria [1]. The antimicrobial activities of phages committed to the cellular lysis of a range of bacterial pathogens have been reported, which include food pathogens such as Campylobacter jejuni [2-5], Escherichia coli [6-8], and various Salmonella enterica serovars [4,9,10]. However, despite the wealth of data regarding the efficacy of phages during intervention studies, relatively few phage-derived products have been developed sufficiently for commercial application. Only in the last few years has there been an extension of lab-based trials into the food production environment, where perhaps the best example is the recognition of the efficacy and the granting of ‘generally recognized as safe’ (GRAS) status to bacteriophages targeting Listeria monocytogenes by the United States Food and Drug Administration [11]. There are two products of note now available commercially-List-Shield™ (Intralytix Inc., USA) a phage cocktail comprising virulent phages with broad activity against \( L. Monocytogenes \), and Listex P100™ (EBI Food Safety, Netherlands). Following GRAS classification both List-Shield™ and Listex P100™ are now viewed as safe to

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be applied as food biopreservatives on ready-to-eat foods in the USA. Phage P100 (the active component of Listex P100) was initially characterized at the genetic level and in oral toxicity studies by Carlton et al [12]. These studies showed that P100 had no undesirable genes within its genome, and caused no ill effects when administered to rats. Many studies reporting the efficacy of Listex P100 against L. monocytogenes in various food production settings are now available in the literature [13-15]. If more phage-based applications are to reach standards where they are deemed fit for human/animal consumption then certain matters pertaining to safety must be taken into consideration. For instance, phages that show potential during preliminary studies must adhere to strict criteria if they are to be developed further as antimicrobial agents [16]. Most importantly, the selected phages must not possess genes associated with virulence, or those that may enhance the pathogenic profile of its target [17]. Many examples of phages that are recognized as being involved in such processes are known, for example, the Shiga toxin-encoding (Stx) phages-a key virulence factor of Shiga-toxigenic E. coli (STEC)-are the causative agents of haemolytic uraemic syndrome (HUS), a major contributor to disease associated with STEC infection [18,19]. Also, temperate phages that have the potential to form lysogens with their host need to be eliminated from trials at the earliest opportunity. Integration into, and excision out of the host genome, can lead to the transfer of genes between the phage and bacterium potentially altering the genetic profile of both [20]. These undesirable traits are most often associated with phages belonging to the Podoviridae (icosahedral head with a short non-contractile tail e.g. Salmonella phage P22) or Siphoviridae (icosahedral head with a flexible non-contractile tail e.g. phage λ) families. However, members of the Myoviridae (icosahedral head and contractile tail e.g. phage T4) are more often associated with an obligately lytic lifecycle. All of these morphological characteristics can be easily discerned when phages are viewed under a transmission electron microscope, although assumptions as to the genetic nature of a phage cannot be made on morphological characteristics alone. Whole genome sequencing of phage isolates intended for use as biocontrol agents is now considered to be the ‘gold standard’ in terms of transferring phage-based applications from the laboratory to everyday use.

Here we report the complete genome sequence of ΦSH19, a lytic bacteriophage adapted to infecting a number of different S. Typhimurium serovars. ΦSH19 has shown great potential as a biocontrol agent against S. Typhimurium U288, the most prevalent serovar found in UK pig production premises [21]. S. Typhi phage Vi01 was the first of a recently-proposed new lineage of Myoviridae to be described. Genetic analyses of the ΦSH19 genome reveal that it is a close relative of phage Vi01, in terms of both DNA and protein sequences. However, phage Vi01 appears to be restricted to infecting S. Typhi, possibly due to the presence of a virulence (Vi) capsule antigen-degrading acetyl esterase domain incorporated into one of its three tail spikes [22]. The other completely sequenced member of the Vi01-like phage lineage is SboM-AG3 that is restricted to the infection of Shigella spp. [23]. No genes associated with either toxicity or lysogeny have been found within the ΦSH19 157,785 bp circular dsDNA genome, nor in any of the Vi01 family of phages. A lack of virulence associated genes in ΦSH19 will allow its use as a biocontrol agent, aimed at reducing S. Typhimurium entering the food chain, and in particular S. Typhimurium U288 from pork production.

Results
Bacteriophage ΦSH19 characterization
Bacteriophage ΦSH19 was originally isolated from pig intestinal contents and has specific activity against S. Typhimurium serovars [24]. Morphologically the phage has an icosahedral head with a contractile tail indicating it to be a member of the Myoviridae (Figure 1). A comparison of the lytic profiles of phages ΦSH19 and Vi01 against a panel of S. enterica Serovars is given in Table 1. The data shows that as expected all the S. Typhimurium strains tested are refractory to infection by Vi01, whereas S. Typhi BRD948 likewise displays immunity towards infection by ΦSH19 but susceptibility to Vi01.

Genome analysis
The ΦSH19 genome was sequenced from sonicated DNA fragments using the Roche 454 GS FLX platform...
Table 1 Host range assays of \( \Phi \)SH19 and Vi01 (+ = lysis and – = no lysis).

| Salmonella                  | \( \Phi \)SH19 | Vi01 |
|-----------------------------|----------------|------|
| S. Typhimurium U288         | +              | -    |
| S. Typhimurium DT104 WT     | +              | -    |
| S. Typhimurium DT104 NCTC 13348 | -           | -    |
| S. Typhimurium WT (Rawlings) | +             | -    |
| S. Typhimurium WT (Turner)  | +              | -    |
| S. Typhimurium LT2          | +              | -    |
| S. Typhi BRD948             | -              | +    |
and an endo-α-sialidase gene of coliphage K1F (76% identical from bases 351-485). Aside from these alignments, which all span a region covering the first 485 bases of tsp1, no homologies were found in the database for the region covering bases 485-1932. Alignment of the tsp2 gene (2175 bp) reveals that only Vi01 and SboM-AG3 have similar sequences within the database. As with tsp1, the homologies all span the start of the gene: 85% identity (bases 1-723) with Vi01 orf 171c (Vi01 Tsp2-maturation/adhesion protein) and 70% identity (bases 1-360) with SboM-AG3 orf 00210 (SboM-AG3 Tsp2). The latter portion of the tsp2 gene does not align with any sequences in the database. BlastN searches involving the tsp3 gene (2097 bp) show that the sequence is highly similar to the Det7 tail spike gene (85% identity from bases 1-782). To a lesser extent, tsp3 shares sequence homology (84% identity between bases 1-461) with Vi01 orf 172c (Vi01 Tsp3) and orf 170c (69% identity between bases 1-179). Apart from these short regions of homology, there are no further identities between tsp3 and Vi01. Interestingly, no nucleotide sequence homology was found between tsp3 and SboM-AG3. Bases 481-667 show a conserved sequence that is shared between several phage genomes in the database. Sequence alignments over this region indicate 74-77% shared identities between various Salmonella phages including P22, ST104, ST64T, ST160, SE1, and some of the S. Enteritidis typing phages (SETP) [25]. Interestingly, the sequence is also present in prophage tail spikes of S. Typhimurium D23580, S. Typhimurium T000240, S. Heidelberg SL476, and S. Paratyphi A (strain AKU 12601). As was the case for tsp1 and tsp2 no homologous sequences are found to match the latter region of the tsp3 gene.

Translation of the tsp gene sequences indicate that Tsp1 is a 643 amino acid protein (predicted MW ~ 68.9 kDa), Tsp2 is comprised of 724 amino acids (predicted MW ~ 78.2 kDa), and Tsp3 698 amino acids (predicted MW ~ 75.8 kDa). Pfam domain searches for each tail spike indicated significant domain matches for Tsp2 (Pectate lyase domain-family 3 CL0268) and Tsp3 (P22 tail spike family); however no significant domain
| CDS               | ΦSH19 coordinates & amino acid length | Predicted MW | Pfam domain                                                                 | Amino acid ID with Vi01 | Amino acid ID with SboM-AG3 |
|-------------------|--------------------------------------|--------------|------------------------------------------------------------------------------|-------------------------|-----------------------------|
| RIIA              | 1-2742 (913)                         | 104,992      | None                                                                        | 98%                     | 54%                         |
| RIIIB             | 2775-4358 (527)                      | 58,980       | None                                                                        | 97%                     | 69%                         |
| Putative tail fibre | 5502-6311 (269)                      | 27,826       | Ig-like I-set domain (CL0111)                                               | 86%                     | 86%                         |
| DNA topoisomerase II | 8523-10,436 (637)                  | 71,862       | HATPase c (CL0025) & DNA gyrase B                                            | 96%                     | 90%                         |
| DNA topoisomerase II (medium subunit) | 10,357-11,769 (470)     | 53,939       | DNA topoisomerase IV                                                         | 88%                     | 88%                         |
| Conserved uncharacterized protein                 | 13,309-13,929 (206)                 | 23,005       | Putative Macro domain of ADP-ribose binding module (CL0233)                | 92%                     | 74%                         |
| DexA exonuclease        | 14,263-14,895 (210)                 | 24,438       | None                                                                        | 95%                     | 93%                         |
| dCMP deaminase            | 18,704-19,225 (173)                 | 19,460       | dCMP cytosine deaminase 1 family-cytidine & deoxycytidylate deaminase Zn-binding domain (CL0109) | 97%                     | 62%                         |
| Conserved uncharacterized protein | 19,227-19,634 (135)               | 15,378       | Bacterial membrane-flanked domain (DUF304)                                 | 93%                     | 88%                         |
| Head completion protein | 19,845-20,465 (206)               | 24,189       | None                                                                        | 92%                     | 74%                         |
| Putative homing endonuclease | 20,465-21,205 (246)              | 28,205       | GY-YIG catalytic domain (CL0418)                                            | No match                | 34%                         |
| T4-like baseplate tail tube cap          | 21,259-22,279 (322)                | 36,142       | T4 tail cap family                                                         | 100%                    | 94%                         |
| Baseplate wedge subunit       | 22,240-22,797 (185)               | 21,574       | Phage Gp53 family                                                          | 97%                     | 80%                         |
| Loader of T4-like helicase       | 26,134-26,796 (220)               | 26,253       | T4 helicase N family                                                       | 99%                     | 79%                         |
| DNA ligase                  | 27,281-28,708 (475)                | 53,501       | ATP-dependent DNA ligase domain (CL0078)                                    | 95%                     | 83%                         |
| DNA primase-helicase subunit       | 31,451-32,875 (474)               | 54,192       | DNA helicase N & C terminal domains                                        | 98%                     | 88%                         |
|UvsX (RecA-like protein)          | 33,189-34,274 (361)               | 40,791       | RecA bacterial DNA recombination proteins (CL0023)                         | 72%                     | 78%                         |
| dUTPase                       | 34,789-35,340 (183)                | 21,114       | dUTPase 2 family(CL0231)                                                    | 69%                     | 63%                         |
| Thymidylate synthase               | 35,903-36,943 (346)               | 39,216       | Thymidylate synthase family                                                 | 93%                     | 77%                         |
| DNA end protector               | 39,924-40,268 (234)               | 28,347       | None                                                                        | 100%                    | 91%                         |
| Baseplate tail tube            | 40,488-41,624 (378)                | 42,658       | Phage T4 Gp19 family                                                       | 98%                     | 90%                         |
| ssDNA binding protein          | 41,652-42,683 (343)                | 38,896       | Gp3 DNA binding protein-like domain                                        | 98%                     | 77%                         |
| Late promoter transcription factor | 43,029-43,331 (100)               | 11,175       | None                                                                        | 100%                    | 70%                         |
| Regulatory protein (FmdB family)  | 43,264-43,509 (81)                | 8732         | CXXC CXXC SSSS family containing a Zn ribbon domain (CL0167)                | 96%                     | 88%                         |
| Putative uncharacterized protein  | 43,875-44,394,0 (203)              | 23,739       | RuvC family (crossover junction endonucleosase RuvC)                      | 100%                    | 83%                         |
| Baseplate hub subunit           | 45,471-46,274 (267)                | 30,186       | T4 baseplate family                                                        | 96%                     | 67%                         |
| Tail-associated lysozyme            | 46,781-48,404 (541)               | 59,023       | Gp5 OB family & CHAP domain (CL0125) associated with peptidoglycan hydrolysis | 98%                     | 87%                         |
| Baseplate wedge protein        | 48,471-48,851 (126)                | 14,124       | GPW Gp25 family (gene 25-like lysozyme)                                     | 97%                     | 87%                         |
| NrdB                            | 50,229-51,332 (367)                | 42,209       | Ribonucleotide reductase small chain domain (CL0044)                        | 99%                     | 89%                         |
| NrdA                            | 51,403-53,730 (775)                | 88,137       | ATP-cone domain, ribonucleotide reductase IgN all alpha domain & IgC barrel domain | 94%                     | 89%                         |
| PhoH-like phosphate starvation-inducible protein | 53,764-54,603 (279)          | 31,567       | PhoH-like protein family (CL0023)                                           | 100%                    | 88%                         |
| Peptidoglycan binding protein       | 54,711-55,505 (264)               | 28,815       | Peptidoglycan binding protein domain (CL0244) & protein of unknown function (DUF3380) | 98%                     | 86%                         |
| DNA primase subunit             | 56,712-57,776 (354)                | 41,386       | None                                                                        | 97%                     | 77%                         |
| Conserved uncharacterized protein | 63,656-64,273 (205)               | 22,902       | T4 RegB endonucleosase family (CL0037)                                     | 98%                     | 79%                         |
| NrdA.1     | 64,978-65,313c (336) | 12,934 | None | 94%  | 92%  |
|------------|----------------------|--------|------|------|------|
| Recombination endonuclease subunit | 65,622-67,955c (777) | 88,531 | None | 98%  | 72%  |
| Recombination protein subunit | 67,958-69,073c (371) | 43,030 | None | 98%  | 91%  |
| σ factor for late transcription | 69,060-69,854c (264) | 30,775 | None | 90%  | 77%  |
| Putative homing endonuclease | 69,854-70,576c (240) | 27,188 | None | No match | 36%  |
| RNase H | 70,587-71,114c (175) | 19,878 | RNase H domain (CL0219) | 97% | 71% |
| ATP-dependent helicase | 71,921-73,522c (533) | 60,690 | None | 99% | 86% |
| DNA binding protein | 73,778-74,056c (92) | 9728 | Bacterial DNA-binding protein domain | 100% | 80% |
| Conserved uncharacterized protein | 74,140-74,931c (263) | 28,556 | SPFH domain/Band 7 family (CL0433) | 98% | No match |
| Superinfection exclusion protein | 75,137-75,457c (106) | 12,562 | None | 95% | No match |
| ImpD | 75,376-76,407c (223) | 25,665 | None | 95% | 74% |
| Acyl carrier protein | 80,335-80,687c (110) | 12,162 | Phosphoantetheine (PP)-binding family (CL0314) | 92% | 61% |
| Conserved uncharacterized protein | 84,506-84,925c (139) | 15,622 | Protein of unknown function (DUF3268) | 90% | 84% |
| Putative RegA translational repressor | 89,926-90,390c (154) | 18,078 | Bacteriophage translational regulator | 99% | 90% |
| Clamp loader for DNA polymerase | 90,420-90,842c (140) | 16,200 | None | 98% | 76% |
| Gp44 sliding clamp holder | 90,847-91,836c (329) | 37,214 | ATPase family (CL0023) | 99% | 87% |
| Gp4S sliding clamp holder | 91,914-92,582c (222) | 24,510 | Gp4S sliding clamp C terminal | 99% | 82% |
| Putative type III restriction enzyme (RE) | 93,293-94,792c (499) | 57,802 | Type III RE subunit (CL0023) & helicase conserved C terminal domain (CL0023) | 95% | 81% |
| Conserved uncharacterized protein | 94,822-95,568c (248) | 28,911 | PD-(D/E)XK nuclease superfamily | 97% | 86% |
| Putative UvsY | 95,568-96,023c (151) | 17,991 | UvsY protein family (recombination, repair, & ssDNA binding protein) | 93% | 85% |
| Tail completion protein | 96,062-96,559c (165) | 18,542 | T4 Gp19 family | 98% | 80% |
| Major capsid protein | 101,588-102,910c (440) | 48,059 | Gp23 major capsid protein family | 98% | 84% |
| Prohead core scaffold protein | 103,002-103,841c (279) | 30,898 | None | 96% | 74% |
| Prohead protease | 103,888-104,556c (222) | 24,482 | Peptidase U9 family (CL0201) | 99% | 92% |
| Portal vertex protein of the head | 105,102-106,784c (560) | 63,094 | T4 Gp20 family | 99% | 81% |
| Tail tube protein | 106,852-107,385c (177) | 19,912 | T4 Gp19 family | 100% | 82% |
| GIY-YIG endonuclease | 107,416-107,874c (152) | 17,316 | GIY-YIG catalytic domain endonuclease family (CL0418) | 95% | 32% |
| Tail sheath protein | 107,933-109,828c (631) | 68,439 | Phage sheath 1 family | 98% | 92% |
| Large terminase subunit | 109,881-112,091c (736) | 84,547 | Terminase 6 family | 95% | 85% |
| Small terminase subunit | 112,072-112,752c (226) | 24,825 | DNA packaging family (terminase DNA packaging enzyme) | 98% | 74% |
| Proximal tail sheath stabilization | 112,755-113,453c (232) | 29,679 | None | 98% | 80% |
| Gp14 neck protein | 113,456-114,097c (213) | 24,759 | T4 neck protein family | 100% | 82% |
| Gp13 neck protein | 114,400-115,209c (269) | 31,142 | None | 99% | 87% |
similarities were identified for Tsp1. Betawrap analyses of the amino acid sequences of each tail spike from FSH19, Vi01, and SboM-AG3 shows that each of these proteins potentially have the ability to form right-handed parallel β-helical structures. A comparison of each FSH19 tail spike protein sequence with those of Vi01 and SboM-AG3, and other homologous proteins in the database revealed the N-terminal residues (1-130) of FSH19 Tsp1 are highly conserved with the corresponding regions in Vi01 Tsp1 (78% ID), SboM-AG3 Tsp1 (75% ID), and to a lesser degree in Vi01 Tsp3 (56% ID). This N-terminal homology is also shared with the
Salmonella phage Det7 tail spike (56% ID), and a tail fibre protein from Enterobacter phage Ecpl (42% ID). A sequence motif present in FSH19 Tsp1 G-G-V-G-L-G-A-W (beginning at residue 143) possibly signifies the boundary at which the catalytic domain of the tail spike begins. No such sequence motif is present in Vi01 Tsp1, however similar sequence motifs are present at analogous positions within SboM-AG3 Tsp1 (G-G-L-S-S-N-W) and Vi01 Tsp3 (G-G-V-G-T-G-A-W). ClustalW2 alignments of phage tail-associated proteins containing derivatives of these motifs at their N-terminal boundaries are shown in Figure 4A. The C-terminus of FSH19 Tsp1 (residues 300-643) produced no significant alignments with any known proteins. BlastP analyses of Vi01 Tsp1 and SboM-AG3 Tsp1 was performed to identify any functionally-related proteins. However, as with FSH19 Tsp1, Vi01 Tsp1 produced no significant alignments over the C-terminal region with any protein sequences in the database.

Alignment of FSH19 Tsp2 with Vi01 Tsp2 (matura-
tion/adhesion protein) and SboM-AG3 Tsp2 shows a high degree of conservation from residues 1-160. At this point SboM-AG3 sequence homology (73% ID over residues 1-162) breaks down with no further identity to either Vi01 or FSH19 Tsp2. Residues 100-403 in FSH19 Tsp2 show a weak but significant relationship with a phage structural protein/putative tail fibre (orf 00213) present in SboM-AG3. For FSH19 Tsp2 and Vi01 Tsp2, homology continues over the first 276 residues (86% ID) at which point the two sequences completely diverge from each other. Another region of low homology (residues 100-316) is also found with a putative tail fibre protein encoded in the Vi01 genome (Vi01 orf 173c). A pectate lyase protein domain family (family 3-CL0268) spanning residues 291-549 was identified during Pfam analysis of FSH19 Tsp2. The presence of this motif was further confirmed during BlastP analysis, with a number of alignments being made with various glycoside degrad-
ing enzymes from a wide range of microorganisms. No significant alignments with the C-terminus of FSH19 Tsp2 were identified (residues 557-724).

FSH19 Tsp3 alignment with Vi01 Tsp3 shows a high degree of sequence conservation between their N-termi-
nal residues 1-154 (86% ID). However, the correspond-
ing sequence in SboM-AG3 Tsp3 shows similarity over the first 19 residues before a deletion of 63 amino acids, after which the homology is restored between the three tail spike sequences. Interestingly, SboM-AG3 Tsp1 shows a higher degree of conservation with FSH19 Tsp3 over residues 1-139 (46% ID) than does SboM-AG3 Tsp3. The boundary motif G-G-V-G-L-G-A-W appears in FSH19 Tsp3 at residues 143-150 with analogous sequences in Vi01 (G-G-V-G-T-G-A-W) and SboM-AG3 (G-G-V-S-S-S-A-W), after which the sequence homologies between all three tail spikes break down. Pfam domain analysis of FSH19 Tsp3 identified a protein domain (residues 159-698) with significant homology to the P22 tail spike family (PF09251). BlastP analysis produced alignments with Det7 (the first reported Myovirus with a podoviral tail spike), P22, and a number of P22-like phages. The highest degree of homology was found for the tail spike of Det7 (77% ID over the first 300 residues). The sequence motif marking the domain boundary in FSH19 Tsp3 (G-G-V-G-L-G-A-W) is conserved in Det7. P22 tail spike-like sequences feature three conserved residues that function as catalytic components of the endo-rhamnosidase activity associated with P22 tail spike protein [26,27]. These residues are located in the substrate binding cleft of the P22 tail spike and are conserved in FSH19 Tsp3 (Figure 4B: Glu-359, Asp-392 and Asp-395).

Discussion

Bacteriophage FSH19, a candidate for use as a biocon-
trol agent against Salmonella Typhimurium, was sub-
jected to whole genome sequencing. This process is now
viewed as a pre-requisite to using phages as therapeutic
agents, especially if they are to be introduced into a
food production environment. The presence or absence
of genes associated with toxicity or lysogeny will ulti-
mately govern whether or not a potential phage candi-
date is safe for commercial use. FSH19 was found to
contain no undesirable genes in its 157,785 bp genome
making it an excellent candidate for such applications.
Also, FSH19 constitutes a new addition to the Vi01-like
phage family (along with S. Typhi phage Vi01 and Shi-
gella phage SboM-AG3). In many respects FSH19 is
quite similar to Vi01 with the addition of a putative
homing endonuclease and a putative uncharacterized
protein. Structural genes and those associated with mor-
phology of the phage particle are highly conserved
between FSH19 and Vi01, and to a lesser degree with
SboM-AG3. Analysis of the tRNA genes encoded by all
three phages, and their location within each genome
indicates that some of them are possibly derived from a

Table 3 Putative phage regulatory elements identified by Phire analysis.

| Putative phage regulatory elements | From | To | Sequence |
|-----------------------------------|------|----|----------|
| 56,300                            | 56,319|    | TACCCTATGGTAAATCCCT |
| 79,926                            | 79,945|    | CGGCTATGGTATTCCCTT |
| 99,487                            | 99,506|    | TGGCTATGGTACCTCTCTT |
| 101,431                           | 101,450|   | GTCCCTATGGTATTCCCTT |
| 104,872                           | 104,891|   | TTTCCTATGATGTCCTCTCT |
| 109,822                           | 109,841|   | TTGCATAATACATTCCCTT |
| 144,434                           | 144,453|   | TTGCCTATGGTATTCCCTT |
Figure 4  A-C ClustalW2 alignments of sequence motifs in tail-associated proteins (4A), the active site of P22-like tail spikes (4B), and the C-terminal region of P22-like tail spikes-yellow-sequence motif (residues 585-596 in P22) associated with intertwined region, green-five-stranded β-sheet and three-stranded β-sheet (residues 601-664 in P22), and red-regions lacking homology in ΦSH19 Tsp3 (4C).
common ancestor. Transmission electron microscope images of FSH19 (Figure 1), Vi01 and SboM-AG3 indicate that their morphologies are quite similar. The intricate ‘chandelier-like’ arrangements of tail spikes attached to the baseplate are visible for all three phages [22,23]. It seems likely that there are more Vi01-like phages (such as Escherichia phage PhaxI) capable of infecting a diverse range of bacterial pathogens to be discovered for which there are commercial applications.

The modular structure of the three tail spikes found in FSH19 is potentially the main driving factor behind host-range determination for this phage. Although no function can at present be assigned to Tsp1, the other two tail spikes contain defined protein domains that provide evidence as to their mode of action. For Tsp2, the pectate lyase domain indicates that this protein may well function to modify or cleave glycoside bonds. The pectate lyase family domain (CL0268) notably contains proteins with pectate/pectin lyase and pectin methylesterase activities. The pectate/pectin lyases are a well-characterized family of proteins principally involved in microbial plant pathogenesis. Their primary mode of action involves the eliminative cleavage of α-1,4 linked galacturonosyl residues of pectins that are components of the middle lamella of plant cell walls. The three dimensional structure of Erwinia chrysanthemi EC16 pectate lyase C (PеЬC) was first solved by Yoder et al. [28] and is representative of a family of proteins containing right-handed parallel β-helical structures with the flexibility to allow protein loops from the stacked coils to add functionality, for example the formation of the active site clefts that support enzyme catalysis [29,30]. Using a similar base architecture pectin methylesterase acts to de-esterify pectin to pectate. Members of the pectinolytic protein family are represented in prokaryotic and eukaryotic microorganisms, and also function in plants to remodel cell walls [29]. Divergence from the ancestral sequence over time has allowed different microorganisms to target a range of pectin-like substrates, while the overall structure has been maintained [30]. It is of little surprise that phages have evolved the ability to acquire these proteins and utilize their polysaccharide modifying properties. In the case of FSH19 Tsp2 it could be postulated that the target is the α-D-galactose (1-4) α-D-mannose linkage found in the S. Typhimurium O-antigen trisaccharide repeat. Many pectate tail-associated proteins have been found to contain right-handed parallel β-helical structural domains, for example phages P22 [31], Si6 [32], HK620 [33] and Det7 [27]. Moreover the presence of a pectate lyase-type structure in a phage tail spike protein has previously been reported for K5 lyase (KflA) of coliphages K5a and K1-5. KflA targets and degrades the capsular polysaccharide of E. coli K5, thus allowing the phage access to outer membrane receptors [34]. Whilst the precise identity of the polysaccharide target of FSH19 Tsp2 is at present unknown, it is possible that this tail spike plays a major role in host-range determination. Plaque assays using phages Vi01 and FSH19 against a panel of S. Typhimurium and S. Typhi BRD948 indicate that these phages are specific for their host (Table 1). This is perhaps not surprising since Vi01 orf 171c (Vi01 Tsp2) possesses a 9-0-acetyl esterase/acytelyxylan esterase domain (residues 343-445; DUF303) that targets the acetyl-modification of the S. Typhi Vi capsule [22]. The pectate lyase domain found in FSH19 Tsp2 is more than likely involved in the degradation of a polysaccharide present on the surface of S. Typhimurium U288. Anany et al. [23] also report that SboM-AG3 is Shigella-specific and is unable to form plaques on lawns of any of the Salmonella, Escherichia, and Listeria strains tested.

A protein domain composed of parallel right-handed β-helices is also found in FSH19 Tsp3-the P22 tail spike domain. Many tail spike proteins possess an N-terminal binding domain involved in attaching the protein to the virion head/tail structure (the sequence of which is often highly conserved between phages with similar morphologies), a central catalytic domain (containing the trimeric parallel right-handed β-helices), and a C-terminal trimerization domain (involved in stabilization of the trimeric tail spike). The trimeric β-helices that form the catalytic domain bind and cleave polysaccharides present in bacterial lipopolysaccharide (LPS). P22 tail spike (Gp9) utilizes this endo-rhamnosidase activity to degrade the Salmonella O antigen [35]. Cleavage of α-L-rhamnose (1-3) α-D-galactose found in the trisaccharide-repeats of S. Typhimurium LPS takes place on the external surface of the tail spike. The structure and arrangement of the parallel β-helix tail spike domains result in a solvent-exposed exterior that features the proposed catalytic residues Glu-359, Asp-392, and Asp-395 [27,36]. The active site residues and their sequence environments are conserved in FSH19 Tsp3, and therefore can be postulated to operate in a manner akin to the P22 tail spike and its relatives. Thus, there is a possibility that FSH19 Tsp3 acts in two distinct ways. Firstly, FSH19 Tsp3 may function as an initial step towards irreversible adsorption of the phage particle to its host, hydrolyzing the outer S. Typhimurium LPS layer in order to provide access to a previously inaccessible outer membrane receptor. Secondly, the endo-rhamnosidase activity associated with P22-like tail spikes may aid phage progeny in freeing themselves from cellular debris encountered during host lysis [37].

Tsp3 of FSH19 has an N-terminal sequence similar to Vi01 orf 172c (Vi01 Tsp3), SboM-AG3 orf 00207 (SboM-AG3 Tsp1) and Det7 tail spike. These phages are
all Myoviridae of similar morphologies. Therefore the requirements of having to attach the tail spike to similar base plate structures more than likely places constraints on sequence divergence. Likewise, the alignments for the Podoviridae tail spikes show N-terminal conservation between related phage, followed by conserved catalytic and C-terminal domains that are shared between Myoviridae, Podoviridae and Siphoviridae. Interestingly, P22, ST64T, and ST104 as members of the Podoviridae are 98-99% identical over their 667 amino acid tail spike proteins, and similarly Siphoviridae members SETP12 and SETP13 also have almost identical tail spike sequences. Despite that the SETP 12 and 13 phage sequences are markedly different from P22, ST64T, and ST104 over the N-terminal regions; however, they become conserved between residues 140 to 684. As noted above, there appears to be sequence divergence constraints placed on conserved N-terminal regions associated with the myoviruses ΦSH19, Vi01, SboM-AG3 and Det7. Similarly it appears there are structural restrictions imposed on the Podoviridae and Siphoviridae N-terminal tail spike sequences. Similarities between ΦSH19 Tsp3, Det7 and the P22-like sequences begin ten amino acids after the boundary motif G-G-V-G-L-G-A-W that appears in ΦSH19 Tsp3 and Det7 (this motif also signals the divergence of the tail spike sequences of phages Vi01 and SboM-AG3 from ΦSH19 and Det7). ΦSH19 Tsp3 and Det7 Tsp show striking conservation with the P22-like sequences over the catalytic domain, up to and including the active site residues Glu-359, Asp-392 and Asp-395. However, immediately after the active site in ΦSH19 Tsp3 (residues 440-698) sequence conservation breaks down.

For P22-like tail spike proteins, the C-terminal residues 585-596 form an intertwined region that allows extensive hydrogen bonding between subunits, whilst residues 606-664 form a five-stranded and a three-stranded β-sheet region [27]. ClustalW2 alignment of these regions show that all the aligned P22-like tail spikes contain conserved residues at these locations (including the Myovirus Det7), that set them apart from ΦSH19 Tsp3 (Figure 4C). The eight amino acids spanning residues 585-592 (V/G-G-P/A-G-S/T-G-S-A-W) retain a sequence motif similar to that found at the N-terminal boundary of ΦSH19 Tsp3, and the tail-associated proteins aligned in Figure 4A. However, this motif is absent at the C-terminal end of ΦSH19 Tsp3. P22-like tail spike modules may well have descended from a common ancestor with the flexibility to diverge their protein sequences whilst retaining the topology of the right-handed parallel β-helices. It has been proposed that this flexibility has allowed the protein domain to become widespread amongst bacteriophages [33], with the possibility of later domain interchange creating further diversity [38].

In summary, we have sequenced the genome of ΦSH19, a promising candidate for phage biosanitization of S. Typhimurium in the food production environment. The 157,785 bp circular dsDNA genome was found to contain no genes associated with toxicity or lysogeny, which is a pre-requisite for such applications. Analysis of the nucleotide sequence of ΦSH19 revealed only two close relatives in the database: S. Typhi-specific Vi01 and Shigella-specific SboM-AG3 that collectively form the Vi01-like phage family. Major differences were evident between ΦSH19 and Vi01 in three different tail spike proteins. Two tail spikes from ΦSH19 contain protein domains associated with the degradation of polysaccharides common to Salmonella LPS, namely the pectate lyase (Tsp2) and P22 tail spike-like (Tsp3) families of proteins. The acquisition of these domains is the most obvious reason to explain the different host specificities of ΦSH19, Vi01 (seemingly host-restricted due to the presence of a Vi antigen-degrading tail spike protein domain) and SboM-AG3 (whose tail spikes are as yet undefined). Based on amino acid sequence analysis the tail spikes from all three phages appear to form right-handed parallel β-helical structures. This appears to be an evolutionarily-conserved structure for all three tail spikes found in the Vi01-like phage. However, this conservation is coupled to the exchange of individual protein domains within these structures that may ultimately determine host range.

Materials and methods

Isolation of ΦSH19

For phage isolation, sewage effluent was filtered through 0.2 µm Minisart filters (Sartorius Biotech. Cat. No. 16534) and the filtrate collected in sterile universals and stored at 4°C until required. For S. Typhimurium lawn preparation NZCYM broth (Difco Cat. No. 240410) cultures were prepared and incubated overnight at 37°C with shaking. Following this, the overnight culture was used to seed fresh NZCYM broth containing 10 mM MgSO4 (Sigma Aldrich Chemicals Cat. No. M2643) which was then incubated for 2 hours at 37°C with shaking. To molten (tempered to ~ 50°C) NZCYM top agar containing 0.6% Bacteriological Agar No.1 (Oxoid Cat No. LP0011) 500 µL of the required Salmonella was added, followed by 500 µL filtrate, and the mixture was poured onto NZCYM agar plates. The plates were left to set on the bench for 20 minutes before being inverted then incubated overnight at 37°C. Any plaques identified were picked using sterile pipette tips and resuspended in 500 µL SM buffer (50 mM Tris–HCl [pH 7.5], 100 mM NaCl, 8 mM MgSO4·7H2O, 0.01% gelatin, pH 7.5), incubated at 37°C for 1 hour, then serial diluted in SM buffer. A 25 µL volume of each dilution was then added to Salmonella/NZCYM top agar, and lawns were prepared
as described above. This process was repeated three times in order to obtain a single clonal isolate of \( \Phi S H 19 \).

**Phage host range assays**

The ability of phages \( \Phi S H 19 \) and Vi01 to lyse various \( S. \ Typhimurium \) serovars and \( S. \ Typhi \) BRD948 (kindly provided by D. Pickard Wellcome Trust Sanger Centre, UK) was determined as follows. \( S. \ Typhimurium \) and \( S. \ Typhi \) BRD948 top agar lawns were prepared as described above, with the exception of the latter being grown on supplemented minimal media as described by Tacket et al. [39]. To each bacterial lawn triplicate 20\( \mu \)L volumes of \( \log _{10} \) PFU mL\(^{-1} \) dilutions of the required phage were applied. Following a sufficient drying period, plates were inverted and incubated overnight at 37°C. The following day, each plate was observed for lysis and plates were inverted and incubated overnight at 37°C. The results are shown in Table 1.

**Transmission electron microscopy**

A freshly-prepared high titre phage suspension of \( \Phi S H 19 \) was sedimented at 34,900 \( \times \) g for 2 hours (4°C). Following centrifugation, the supernatant was decanted and each phage pellet was washed twice with 0.1 M ammonium acetate for 1 hour at 25,000 \( \times \) g. The wash solution was discarded and 2 mL SM buffer added to each centrifuge tube. Phage pellets were recovered following overnight incubation at 4°C with gentle shaking. A small drop of washed phage suspension was spotted onto a carbon-coated copper mesh grid and allowed to sit for 3 minutes. Excess phage suspension was then removed with filter paper. For negative staining one drop of phosphotungstic acid [pH 7.4] was added to each grid, and excess stain was removed one minute later with filter paper. Each grid was then covered and allowed to dry for 15 minutes. Images were taken with a Fei Tecnai Biotwin TEM (Fei Company, USA).

**\( \Phi S H 19 \) DNA extraction and sequencing**

A high titre suspension of \( \Phi S H 19 \) (~ \( 10^{10} \) PFU mL\(^{-1} \)) was subjected to a single-step caesium chloride (CsCl) purification procedure as follows. CsCl (Melfords Cat. No. MB1006) was added to the high titre phage suspension to a final concentration of 0.75 g mL\(^{-1} \). Samples were then subjected to ultracentrifugation at 264,000 \( \times \) g for 24 hours in a Beckman TL100 ultracentrifuge (15°C). Extraction of the band containing purified highly-concentrated \( \Phi S H 19 \) was then performed with a sterile 20-gauge hypodermic needle, and the band was subsequently collected in a sterile tube. Residual CsCl was removed from the sample using an Amicon\textsuperscript{®} Ultra-0.5 30 kDa MWCO centrifugal filter device (Millipore Cat. No. UFC 503008) as follows. Briefly, phage-CsCl solution was added to the column, which was then spun at 17,900 \( \times \) g in a benchtop centrifuge for 30 mins. The column was then washed twice with SM buffer at 17,900 \( \times \) g for 2 minutes. To elute the retained phage, SM buffer was added to the column which was then inverted and placed in a fresh collection tube. The column was then centrifuged at 17,900 \( \times \) g for 10 minutes to recover the phage. \( \Phi S H 19 \) DNA was isolated from the purified stock using the phenol-chloroform extraction method with slight modifications as follows. An equal volume of 10 mg mL\(^{-1} \) Proteinase K (Fisher Scientific Cat No. BPE 1700-500) was added to the CsCl-purified sample, followed by detergent solution (10 ng mL\(^{-1} \) Proteinase K in 50 mM EDTA, 50 mM Tris-HCl [pH 8], 1% N-lauroyl sarcosine (Sigma Aldrich Chemicals, Cat No. L9150)). The solution was then incubated overnight at 55°C. Following this, phage DNA was extracted using phenol: chloroform and ethanol precipitation [5]. The extracted \( \Phi S H 19 \) DNA was purified using a DNA wizard purification kit (Promega, Cat No. A1120) then subjected to whole genome amplification using a REPLI-G kit (Qiagen, UK Cat. No. 150023). Genomic DNA was fragmented to 500 bp using a Covaris S2 sonicator (Covaris Inc., USA) and libraries constructed using a NEBNext DNA Sample Prep Master Mix Set 2 (New England Biolabs Cat. No. E6070S). The libraries were subsequently sequenced using the Roche 454 GS FLX system (Roche Diagnostics, USA).

The fully sequenced \( \Phi S H 19 \) genome was annotated using a combination of CLC Genomics Workbench (CLC Bio, Denmark) and Artemis software [40]. For comparisons between the genomes of \( \Phi S H 19 \), Vi01, and SboM-AG3, ACT software was used [41], and comparison files were generated using the web-based programme DoubleACT http://www.hpa-bioninfotools.org.uk/pise/double_act.html. Nucleotide and protein searches were performed using the Blast search algorithm [42]. For the identification of protein domains of known function, searches of the Pfam database were made [43], whilst protein alignments were generated using ClustalW2 [44]. Betawrap was used to identify potential right-handed parallel \( \beta \)-helices in the tail spike proteins encoded by \( \Phi S H 19 \), Vi01, and SboM-AG3 [45]. For the identification of phage regulatory elements Phire analysis of the \( \Phi S H 19 \) genome was performed [46], and tRNAscan-SE 1.21 was used to identify tRNA genes [47]. The complete annotated \( \Phi S H 19 \) genome sequence has been deposited in the NCBI database (Genbank Acc. No. JN126049).

**Abbreviations**

- gp: gene product, used in the context of functional homologues to coliphage T4 proteins.

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