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BRIEF REPORT

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Structural proteins of *Enterococcus faecalis* bacteriophage ϕEf11

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ABSTRACT

ϕEf11, a temperate *Siphoviridae* bacteriophage, was isolated by induction from a root canal isolate of *Enterococcus faecalis*. Sequence analysis suggested that the ϕEf11 genome included a contiguous 8 gene module whose function was related to head structure assembly and another module of 10 contiguous genes whose products were responsible for tail structure assembly. SDS-PAGE analysis of virions of a ϕEf11 derivative revealed 11 well-resolved protein bands. To unify the deduced functional gene assignments emanating from the DNA sequence data, with the structural protein analysis of the purified virus, 6 of the SDS-PAGE bands were subjected to mass spectrometry analysis. 5 of the 6 protein bands analyzed by mass spectrometry displayed identical amino acid sequences to those predicted to be specified by 4 of the ORFs identified in the ϕEf11 genome. These included: ORF8 (predicted scaffold protein), ORF10 (predicted major head protein), ORF15 (predicted major tail protein), and ORF23 (presumptive antireceptor).

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Introduction

Viruses that infect Enterococci have been known for nearly 90 y (Clark & Clark,[1] Evans[2]). Since then, more than 40 different enterococcal phages have been identified (for a review see reference,[3] and refs.[4-9]). Among these, 22 have genome sequences available. These include: virulent *Siphoviridae* phages OSH-56, IME-EF1, SAP6, BC-611, and EFRM-1 (see refs.[6,8,10-12]), temperate *Siphoviridae* phages EFC-1, F4, V1/7, pp1-pp6, FL1A, FL1B, FL1C, FL2A, FL2B, FL3A, FC1 and ϕEf11 (see refs.[9,13-17]), and virulent *Myoviridae* phages ϕEF24C and EFDG1 (see refs.[18,19]). From these sequence data, the functions of many of the gene products of these phage genomes have been predicted. However, there has been little experimental evidence confirming these predicted functions. Here we present SDS-PAGE and mass spectrometry analysis of virion structural proteins in support of our previous annotation of the genome of *Enterococcus faecalis* bacteriophage ϕEf11.[17]

ϕEf11 is a temperate, *Siphoviridae* morphotype bacteriophage that was originally isolated by induction of a lysogenic *E. faecalis* strain recovered from an infected root canal.[20] Its DNA genome is 42,822 bp in length encoding 65 ORFs.[17] The functions of many of the ϕEf11 open reading frames (ORFs) were predicted based upon sequence similarity with gene products of known function reported for previously characterized bacteriophages. Similar to numerous other phages of low GC Gram-positive bacteria, the genes appear to be arranged in functional modules. Among these 8 functional modules, module 2 (containing ORFs 4 through 10) was predicted to encode the proteins of the virion head structure assembly, and module 3 (containing ORFs 11-20) was predicted to be responsible for tail structure assembly. To extend our

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knowledge of the biology of this phage and to confirm the identity of the genes whose products comprise the phage capsid proteins, we identified the structural proteins of the purified phage virions by SDS-PAGE, and compared their amino acid sequences, as determined by MALDI/TOF/TOF mass spectrometry (MS) analysis, with the predicted amino acid sequences of the proteins encoded by the genes of the head and tail structure assembly modules.

Results and discussion

SDS-PAGE analysis of the dissociated purified phage disclosed 11 well-resolved protein bands ranging in size from 27 to 85 kDa, in addition to several less resolved bands (Fig. 1, Table 1). Of these, the proteins in the 6 most prominent bands (bands 2, 6, 8, 9, 10 and 11) were subjected to MALDI/TOF/TOF MS analysis. The amino acid sequences of the proteins in each of these bands, as determined by the MS analysis, was compared with the amino acid sequences of the

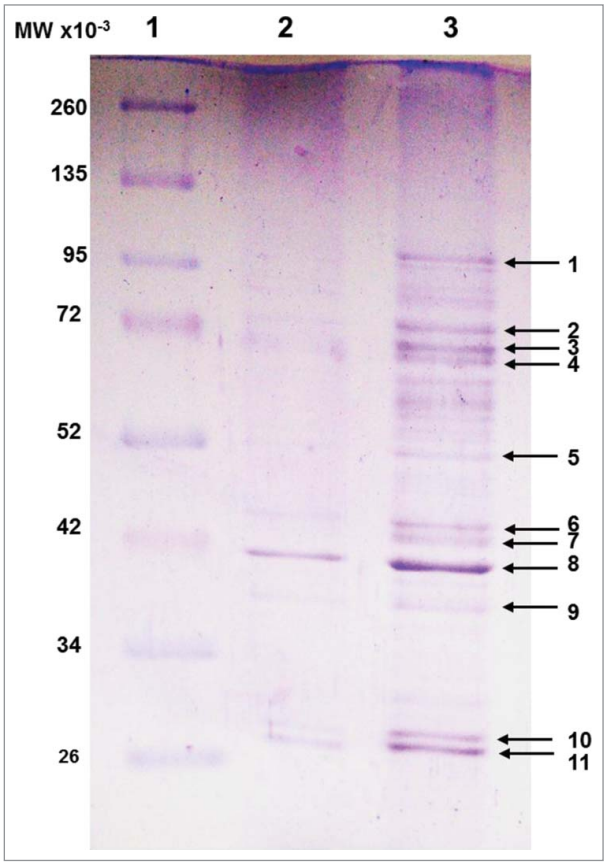

Figure 1. SDS-PAGE analysis of proteins from purified bacteriophage ϕEf11(Δ61-65, FL1C39-44). Lane 1, molecular weight markers (Fermentas Broad Range Protein Ladder; Lanes 2 and 3, dissociated, purified bacteriophage ϕEf11(Δ61-65, FL1C39-44).

Table 1. Summary of phage ϕEf11(Δ61-65, ϕFL1C 39-44) virion proteins predicted from DNA sequence and detected by SDS-PAGE.

Band No./ ORFNo.*	Predicted function of ORF product	MW predicted from DNA sequence	MW determined by SDS-PAGE analysis
2 / 23	Anti-receptor	63,273	73,000 – 74,000
6 / 10	Major head protein	37,324	41,000 – 43,000
8 / 10	Major head protein	37,324	38,000 – 39,000
9 / —			
10 / 15	Major Tail protein	21,542	30,000 – 28,000
11 / 8	Scaffold protein	23,446	27,000 – 27,000

*ORF assignment of the protein in each band made by comparing the deduced gene product amino acid sequence with the amino acid sequences of the peptides detected in each band by MS.

gene products deduced from the genome of ϕEf11.[17] It can be seen in Fig. 2A–D, that the amino acid sequences of 5 of the phage ϕEf11(Δ61-65, FL1C 39-44) virion protein bands (bands 2, 6, 8, 10 and 11) exactly matched the predicted amino acid sequences of the deduced gene products of phage ϕEf11 ORFs 8, 10, 15 and 23 (corresponding to GenBank accession GQ452243.1 loci PHIEF11_0008, PHIEF11_0010, PHIEF11_0015, and PHIEF11_0023, respectively). However, the proteins of both bands 6 and 8 had amino acid sequences corresponding to the predicted gene product of ϕEf11 ORF 10 (locus PHIEF11_0010).

The peptides detected from the protein seen as band 2 of the SDS-PAGE gel had amino acid sequences that were identical to those predicted from the deduced amino acid sequence of the ORF 23 (locus PHIEF11_0023) gene product (Fig 2A), although the observed MW of this protein (ca. 73 kDa) was somewhat larger than that calculated (63.273 kDa) from its predicted amino acid sequence (Table 1). Previously, it was not possible to predict the function of the ORF 23 gene product from its deduced amino acid sequence since there was little sequence similarity to any characterized protein of known function.[17] Furthermore, ORF 23 is located downstream of what we predicted to be the terminal gene (ORF20) of the tail morphogenesis gene module (ORFs 11 through 20). Therefore, in our previous annotation of the ϕEf11 genome, we characterized the ORF 23 gene product as a "hypothetical protein."[17] Subsequently, based on bioinformatics inference to a characterized lactococcal phage tail[21] we initiated infection inhibition studies using the product of the cloned ORF 23, expressed in *Escherichia coli*. These studies, which will be presented in a subsequent report, clearly demonstrated that the ORF 23 gene product blocked

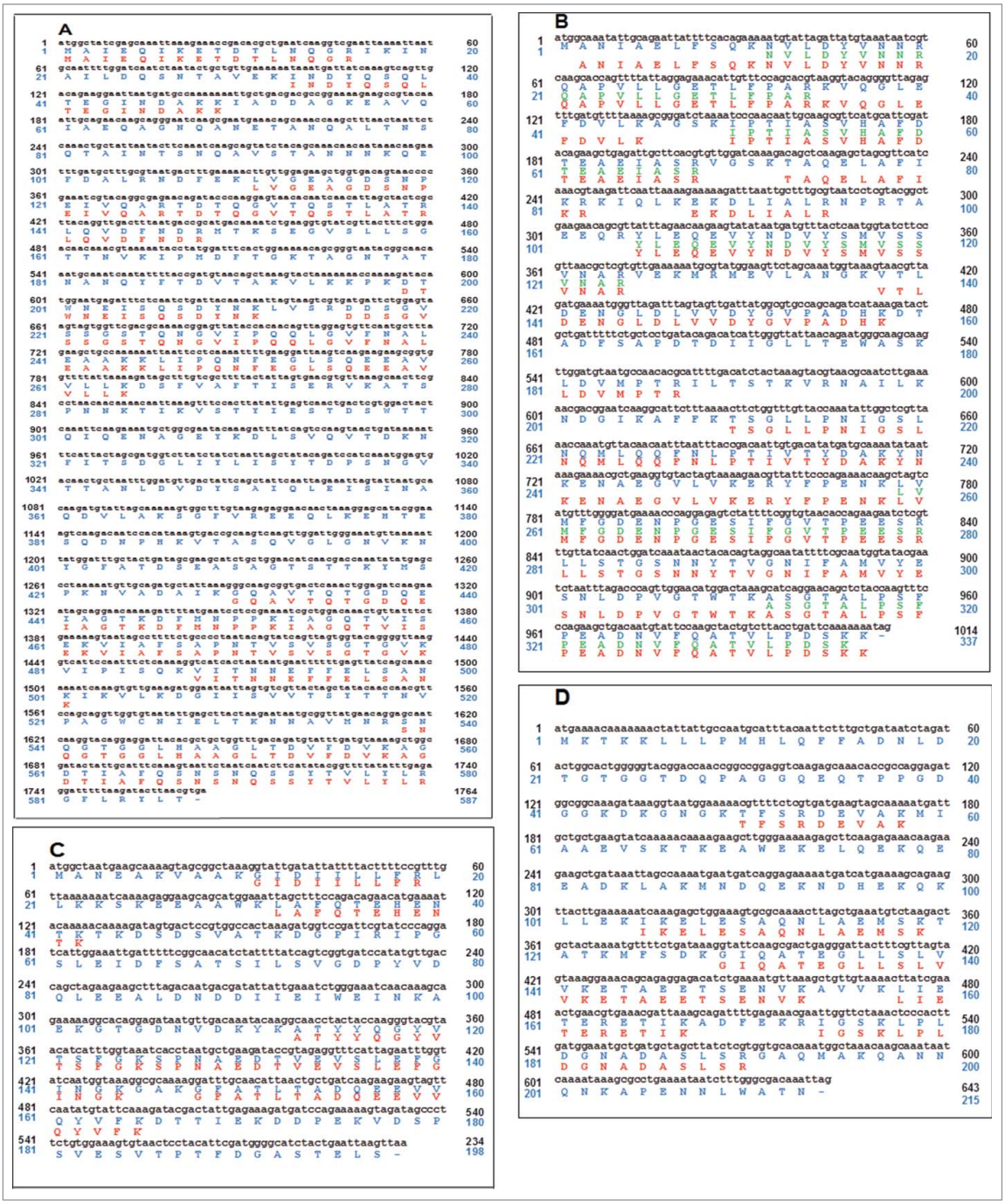

Figure 2. Comparison of deduced amino acid sequences of bacteriophage ϕEf11 gene products with amino acid sequences of peptides detected in MS analysis of SDS-PAGE-separated bacteriophage ϕEf11(Δ61-65, FL1C39-44) virion proteins. Black lettering is the ORF DNA base sequence, Blue lettering is the deduced gene product amino acid sequence, Red or Green lettering is the amino acid sequence of the peptides detected by MS analysis of the material in the bands seen in the SDS-PAGE-separated ϕEf11(Δ61-65, FL1C39-44) virion proteins. (A) ORF23/SDS-PAGE Band 2 protein. (B) ORF10/Bands 6 and 8 proteins (Green is SDS-PAGE band 6 material, Red is SDS-PAGE band 8 material). (C) ORF15/Band 10 protein. (D) ORF8/Band 11 protein.

infection by phage ϕEf11(Δ61-65, FL1C 39-44), and suggested that this protein represents the receptor-binding apparatus of this phage (data not shown). These findings, along with the fact that we now find this protein (band 2) in the SDS-PAGE analysis of the dissociated, purified virion, suggest that ORF 23 codes for a tail structure protein, notably, the receptor-binding protein. Considering the modular nature of the phage genome, these results suggest that the ϕEf11 tail morphogenesis gene module should be extended to include ORFs 21–23 (loci PHIEF11_0021-PHIEF11_0023) (Fig 3).

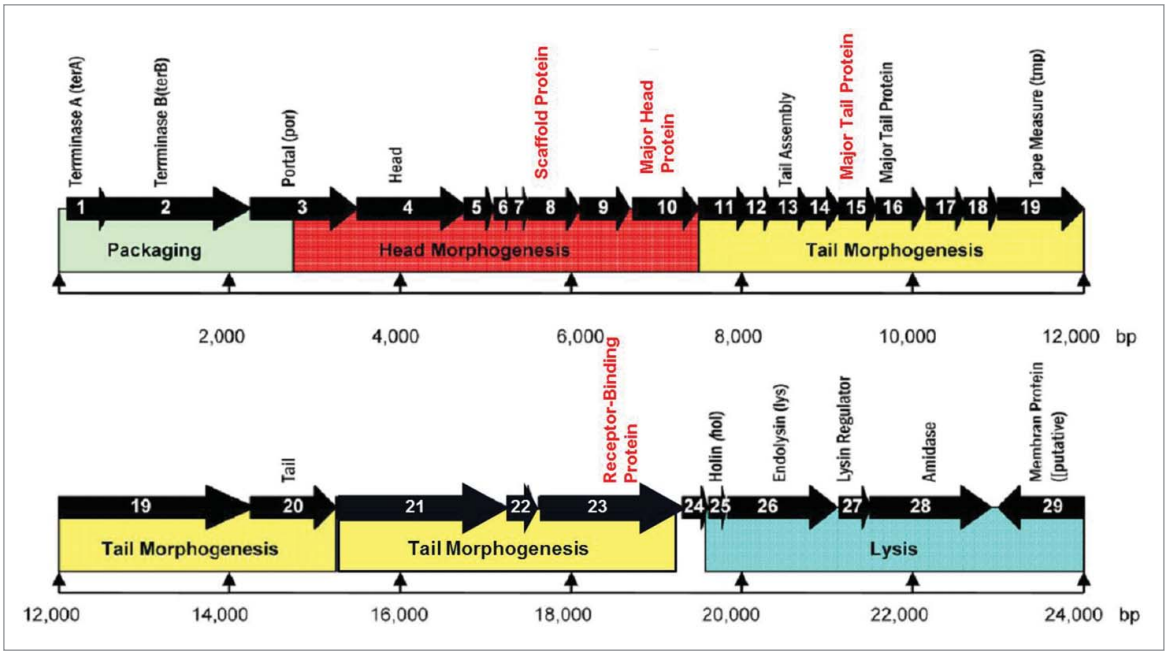

Figure 3. Map of head and tail morphogenesis region of bacteriophage ϕEf11 genome.

Bands 6 (ca. 41 kDa) and 8 (ca. 38 kDa) seen in the SDS-PAGE gels displayed polypeptide fragments with identical amino acid sequences, as detected by mass spectroscopic analysis (Fig. 2B, Table 1). Furthermore, the amino acid sequence of the proteins comprising bands 6 and 8 both precisely matched the deduced amino acid sequence of the gene product of ORF 10 (locus PHIEF11_0010), a protein with a predicted mass of 37.324 kDa, and the predicted function as a major component of the phage head structure (Fig. 3).[17] These data suggest that the 2 proteins seen as band 6 and band 8 in the SDS-PAGE gels are both encoded by the same gene, ORF 10, although one protein (from band 6) exceeded the mass of the other protein (from band 8) by ca. 3 kDa. A similar circumstance has been reported for several other phages (*Bacillus subtilis* phage SPP1,[22] *Listeria monocytogenes* phage PSA,[23] *Lactococcus lactis* phage Q54[24]) in which 2 structural proteins of different masses, detected by SDS-PAGE analysis, were encoded by a single gene. For example, in the case of phage SPP1, 2 bands seen in SDS-PAGE analysis of the phage virion proteins were shown to be gene products of the same ORF; however, one had an observed additional mass of 9.1 kDa.[22] In all these cases, the production of the larger version of each pair of proteins was seen to be due to a programmed translational frameshift resulting in the elimination of a stop codon and the consequent extension of the translated product of the reading frame.[22,23,24] The conditions known to promote a translational reading frame-shift near the 3' terminus of phage mRNA include a region of overlapping synonymous or identical codons just upstream from the stop codon, and the presence of an RNA secondary structure (e.g., a stem-loop) adjacent to the site of the reading frame-shift. The overlapping synonymous or identical codons, forming what is known as a "slippery sequence," allow one base backward slippage of the ribosome in the 5′ direction as it moves along the mRNA. The slippery sequence takes the form of a heptanucleotide with the sequence of X XXY YYZ, where Y is either A or T[25]. If there is any destabilization of the 2 ribosome-bound tRNAs from their codons in the mRNA, the slippery sequence permits bond reformation of the tRNAs to the mRNA, one nucleotide upstream (in the 5′ direction), resulting in a frameshift for all subsequent downstream codons of the ORF. As a consequence, a downstream stop codon may be converted to an amino acyl-tRNA-specifying sequence, resulting in translational readthrough past the original stop codon, and the C-terminal extension of the protein product.[25,26] Such a mechanism was originally reported for retroviruses by Jacks et al,[27] and has been shown to be a common feature in the synthesis of phage major

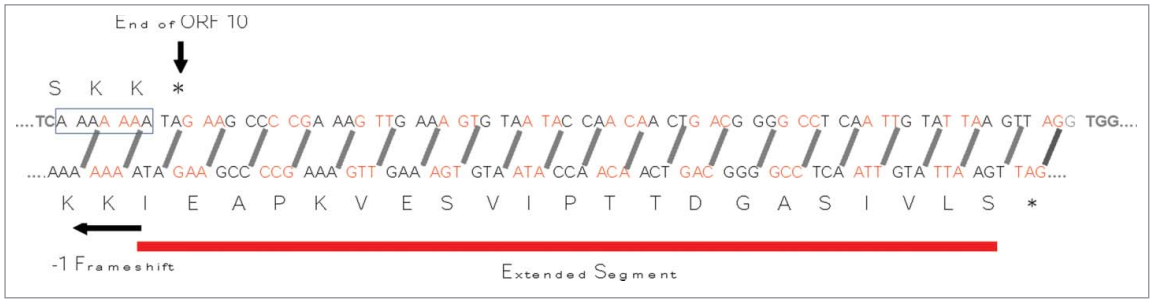

Figure 4. Region of 3′ end of ORF 10. The 0 reading frame is shown in the upper nucleotide sequence. Proposed slippery sequence (AAAAAAA) is indicated by a solid line box. The ORF stop codon is indicated by *. A potential −1 frameshift would result in the nucleotide sequence shown below. In the shifted reading frame, the original stop codon would be converted into 2 amino acid-specifying codons (ATA and GAA), and the protein translation product would be extended by 21 amino acids.

tail proteins of the tailed phages[22,23,26,29,30] as well as in phage capsid head proteins.[31] The RNA secondary structure is thought to positively influence frame-shifting by impeding the progress of the ribosome along the mRNA, thereby allowing more time for realignment of codon and anticodon.[32]

In the case of ϕEf11 ORF10, it can be seen that there are 2 tandem identical (AAA) lysine codons immediately upstream to the ORF10 TAG stop codon (Fig 4). Other phages having slippery sequences composed of AAAAAA include *Mycobacterium thermoautotrophicum* phage ψm2, *Lactococcus* phage c2, *Listeria monocytogenes* phage PSA, *Lactobacillus oenos* phage L10, and *Haemophilus influenza* phage HP1.[28]

There is also the potential for the formation of a stem-loop structure immediately downstream of the stop codon of ORF 10 and the adjacent/overlapping lysine codons, as determined by MFOLD analysis[33] (Fig 5, $\Delta G = -20.10$). These observations are consistent with a potential for a translational frame-shift that would extend the translated gene product by 21 amino acids. As illustrated in Fig. 4, a −1 frameshift would result in a read-through past the original in-frame stop codon, and the extension of the resulting protein by 21 amino acids, increasing the protein mass by 2,106 Daltons. If this occurs in phage ϕEf11 infection during expression of ORF 10, it would explain the observation of the 2 protein bands (bands 6 and 8) seen in the SDS-PAGE analysis differing in apparent mass, but composed of a similar amino acid sequence. However, it should be noted that the MS analysis failed to detect any peptides of the larger version of the protein (band 6) with amino acid sequences that would be expected to be present as a result of the extension of the protein. Therefore, until confirmatory information is available, this interpretation of our results should be considered speculative.

The protein comprising band 10 of the SDS-PAGE-separated ϕEf11 virion proteins had an amino acid sequence that matched that of the deduced gene product of ORF 15 (locus PHIEF11_0015, Fig 2C). Based upon sequence similarities, the ORF 15 gene product was predicted to be a major component of the phage tail structure. [17] The present findings are consistent with this prediction; however, the mobility of the band10 protein seen in the SDS-PAGE gel corresponded to a molecular mass of 28 kDa, which is somewhat larger than the molecular mass predicted

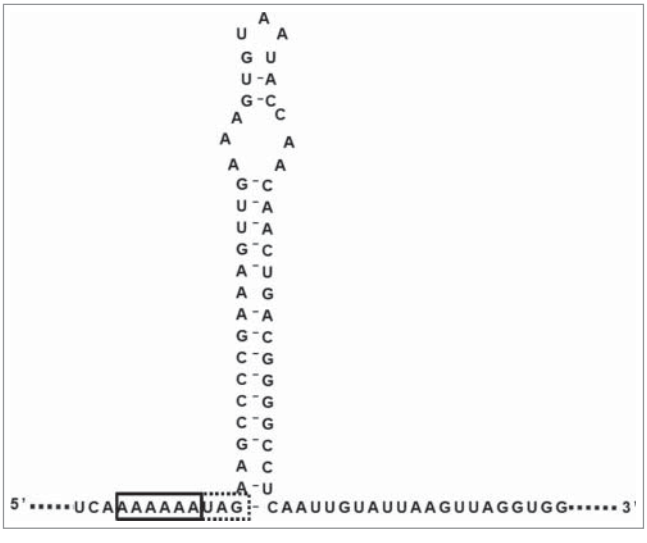

Figure 5. Potential secondary structure of mRNA in region immediately downstream of the proposed slippery sequence and terminal codon of ORF 10, as determined by MFOLD analysis. Initial $\Delta G = -20.10$ kcal/mol. Proposed slippery sequence is indicated by solid line box and 0 frame stop codon is indicated by dotted line box.

for the ORF 15 gene product (21.542 kDa), as determined from the deduced amino acid sequence. Furthermore, the deduced mass of the ORF 15 gene product (21.542 kDa) was less than that deduced for the ORF 8 gene product (23.446 kDa), and yet the putative ORF 15 gene product (band 10) clearly migrated in SDS-PAGE at a rate corresponding to a protein of greater mass than the putative ORF 8 gene product (i.e., band 11, see below). This suggests that either the ORF 15 gene product is post-transcriptionally processed in such a way that it is increased in mass, or the ORF 8 gene product is post-translationally cleaved to result in a decrease in molecular mass. Since both bands 10 and 11 migrated at rates corresponding to greater masses than that predicted from their deduced amino acid sequences, the former alternative seems more likely. There are several examples of phage structural proteins displaying mobilities in SDS-PAGE that correspond to molecular masses that are greater than what would be predicted from their deduced amino acid sequences. For example, the lactococcal phage Tuc2009 scaffold protein (ORF36 gene product Msp2) migrates to a position in SDS-PAGE corresponding to a molecular mass of 40 kDa, although the predicted mass of the ORF36 gene product is 24.5 kDa.[21] Also in Tuc2009, the major head protein (MP4) is encoded by a fusion of portions of ORFs 37 and 39, resulting in an observed molecular mass of MP4 greater than that predicted for either the ORF 37 or ORF 39 gene product alone.[34] Similarly, the computationally-determined masses of the major head and tail proteins (mhp and mtp) of lactococal phage TP901-1 (28.9 and 18.6 kDa respectively) were not in agreement with the masses of these proteins determined by SDS-PAGE (31 and 23 kDa, respectively).[35] It was proposed that the discrepancy between the observed and computationally-calculated masses of each of these proteins was due to a fusion of the genes encoding the mhp and mtp proteins with overlapping genes in different reading frames, resulting in an altered mass and charge in the fused protein.[35] Examination of the alternative reading frames possible within ϕEf11 ORF 15 failed to disclose an overlapping ORF that would permit read-through of an altered ORF 15 stop codon, resulting in an enlarged gene product. Consequently the reason for the apparent increased size of the ORF 15 gene product compared to that predicted from its deduced amino acid sequence may be due to a post-translational modification; however, this remains to be determined.

The protein comprising band 11 displayed an amino acid sequence that was identical to that predicted for the gene product of ϕEf11 ORF 8 (Fig. 2D). Moreover, the molecular mass of this protein as determined by SDS-PAGE analysis (27 kDa) was close to that predicted for the deduced protein gene product of ORF 8 (23.446 kDa). Based upon sequence similarity to deduced characterized proteins in several genomic data base repositories, the ORF 8 gene product was predicted to function as a scaffold protein for phage head structure assembly.[17] The present findings appear to support this contention. Viral scaffold proteins are typically hydrophilic, acidic (pI 4.2–5.0), and small (11.3 kDa-34.0 kDa) in size.[36] The ORF 8 gene product (213 amino acids) has a predicted pI of 5.01, and molecular mass of 23.446 kDa (predicted)−27.0 kDa (observed). Viral scaffold proteins are characterized as proteins that are required for the proper assembly of the viral capsid, but are not present in the mature virion.[36,37] The scaffold proteins are responsible for the correct arrangement of the structural proteins in order to form a procapsid. Following completion of procapsid assembly, the scaffold proteins are typically removed or cleaved prior to DNA packaging and completion of the mature capsid. Therefore, the presence of a scaffold protein in the ϕEf11 virion is somewhat unusual, although there are previous reports of other phages (e.g., *Streptococcus pneumoniae* phage Cp-1 and Lactococcal phage Tuc2009) whose nucleocapsids include at least portions of scaffold protiens.[21,38]

The peptides detected by MS in the SDS-PAGE band 9 material did not display any amino acid sequence homology to any of the deduced ϕEf11 gene products. They were, however, found to be identical in sequence to a protein ("hypothetical protein EF1463") predicted to be a gene product of *E. faecalis* V583. Although the function of this protein (Accession number NP_815184.1) could not be predicted based upon sequence homology, it did possess a conserved domain found in membrane proteins of other bacterial species (*Corynebacterium ulcerans* and *C. pseudotuberculosis*). It is likely that this protein was a contaminant that co-purified along with the *E. faecalis* virions.

In conclusion, peptides having amino acid sequences identical to those of the deduced gene products of

phage ϕEf11 ORFs 8, 10, 15 and 23, were detected in the bands produced by SDS-PAGE analysis of purified phage ϕEf11(δ61-65, FL1C 39-44).

These findings support and strengthen our previous gene function assignments for phage ϕEf11 structural proteins, with the exception that it appears that ORFs 21-23 should now be included in the module of tail structure morphogenesis genes. Specifically, they suggest that ORFs 8, 10, 15 and 23 code for the major head protein, major tail protein, scaffold protein, and the receptor-binding protein, respectively.

Materials and methods

Growth and purification of phage

Phage were grown in 10 Ls of a log phase brain heart infusion broth culture of *E. faecalis* JH2-2. To increase the obtainable phage titer, we used a more virulent recombinant variant of ϕEf11: Phage ϕEf11(Δ61-65, FL1C39-44), which contains 5 genes of an *E. faecalis* phage FL1C-like phage replacing 6 ϕEf11 genes whose predicted functions were related to DNA replication.[39] After incubation at 37°C for 6 h, DNAse (1μg/ml) was added and the culture was incubated at room temperature for an additional hour. The resulting lysate was clarified by centrifugation (GSA rotor at 16,000 × g for 10 min) and the phage was pelleted from the clarified lysate by a series of ultracentrifugations (45 Ti rotor at 150,000 × g for 1 h). After decanting the supernatants, the phage pellets were resuspended overnight at 4°C, in a total volume of 5 ml of SM buffer (0.58% NaCl, 0.2% $MgS0_4$, 0.788% Tris HCl pH7.5, 0.01% gelatin).[40] Portions of the phage suspension were layered onto CsCl (in SM buffer) step gradients having density layers of 1.70, 1.50, 1.40, and 1.15 g/ml. The gradients were centrifuged (SW41 rotor at 110,000 × g for 2 h), and the phage bands, which formed at the 1.40/1.50 g/ml interface were collected and dialyzed against SM buffer at 4°C overnight. Finally, the phage was pelleted (45 Ti rotor at 150,000 × g for 1 h), and resuspended in TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA).

SDS-PAGE

The phage was dissociated in SDS-PAGE sample buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.025% bromphenol blue), and the phage proteins were separated by SDS-PAGE in 13% polyacrylamide gels.[41] Electrophoresis was carried out for at 200 V for 1h, and the phage proteins were visualized by staining with Coomassie Brilliant Blue.

TOF/TOF mass spectrometry

Selected, well-resolved bands were excised from the polyacrylamide gel. The protein in each excised band was eluted in distilled water and subjected to mass spectrometry analysis following procedures described by Tibrewal et al.[42] The eluted proteins were extracted with 30 μl of 1% trifluoroacetic acid followed by C_{18}ZipTip desalting. After mixing with 7 mg/ml α-cyano-4-hydroxy-cinnamic acid matrix in a 1:1 ratio, the peptides were spotted onto a MALDI plate. Peptide analysis was carried out with a 4800 Protomics Analyzer tandem mass spectrometer (Applied Biosystems, ABI, Framingham, MA) in positive reflector mode for acquiring the mass spectra (m/z 800-3,000). Subsequent MS/MS sequencing analysis was conducted on the most intense ions. The identification of the peptides detected in the MS/MS sequencing was accomplished by comparing the combined MS and MS/MS spectra with the sequences in the swiss-prot database using a MASCOT search engine.

Abbreviations

GC	guanine/cytosine
MALDI/TOF/TOF	matrix-assisted laser desorption ionization-time of flight-time of flight
MS	mass spectrometry
ORF	open reading frame
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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