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Bacteriophages induced from lysogenic root canal isolates of *Enterococcus faecalis*

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Introduction: Bacterial viruses play crucial roles in the pathogenesis of many systemic diseases. They are known to inhabit the oral cavity, both as free virions and as prophages in lysogenic bacterial strains; however, there has been no report of bacteriophages in endodontic infections. In this study, we sought to detect, isolate, and describe temperate bacteriophages harbored by *Enterococcus faecalis* strains isolated from endodontic infections.
Methods: Ten *E. faecalis* strains were isolated from root canals of teeth undergoing retreatment following unsuccessful endodontic therapy. Mitomycin C was used to induce any prophages present in the bacterial isolates. The induced phages were purified and examined using electron microscopy. The DNA extracted from one of the phage isolates was subjected to restriction endonuclease digestion and agarose electrophoresis analysis.
Results: Lysogeny was demonstrated in 4 of the 10 *E. faecalis* strains. Three of the lysogenic strains yielded phages exhibiting a Siphoviridae morphology, with long, non-contractile tails 130 nm in length, and spherical/icosahedral heads 41 nm in diameter. The virus induced from the fourth lysogenic *E. faecalis* strain had a contractile tail characteristic of Myoviridae. Restriction endonuclease analysis of *Nsi*I and *Nde*I DNA fragments from one of the Siphoviridae phage isolates (phage ϕEf11) indicated a genome size of approximately 41 kbp.
Conclusion: This is the first report of lysogenic bacteria and their inducible viruses in infected root canals.

Key words: bacteriophage; *Enterococcus faecalis*; root canal

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The importance of *Enterococcus faecalis* in endodontic infections has been suggested by several lines of evidence. For example, numerous studies have shown that *E. faecalis* is the most frequently isolated species, often as a monoinfection, from root canals of endodontically treated teeth with persistent apical periodontitis (12, 25, 31, 32, 42, 43). This organism is known to survive a wide variety of extreme environmental conditions (16), and has been shown to be resistant to the calcium hydroxide treatment commonly used in the course of endodontic therapy (19, 40). It persists in experimental infections of root canals as monoinfections (15, 40), and can survive for long periods under nutritional deprivation (17, 37). Endodontic *E. faecalis* isolates have been shown to be uniformly positive for the presence of genes for several virulence factors such as gelatinase (*gelE*), aggregating substance (*asa*), adhesin factor (*EfaA*), and adhesion of collagen (*ace*), but vary with respect to the presence of other virulence-associated genes such as *Enterococcus* surface protein (*Esp*) and cytolysin A (*cylA*) (38). Virulence genes have also been detected in *E. faecalis* strains isolated from a variety of non-oral sources such as endocarditis, sepsis, and biliary stents (9). Furthermore, examination of endodontic *E. faecalis* isolates revealed the presence of plasmids in 25 of 33 strains (36). The finding that endodontic isolates of *E. faecalis* commonly harbor plasmids may be of considerable significance because a variety of virulence-related genes are known to be carried on plasmids (11).

In addition to plasmids, extrachromosomal genetic information in the form of bacteriophages can also markedly alter the virulence potential of the bacteria they infect. Genes located on bacteriophage chromosomes can encode proteins that act as bacterial toxins, as well as proteins that can alter host cell antigenicity, capsule production, and adherence factor

production, and enhance serum resistance (for review see Refs 4, 47). Despite the fact that the *E. faecalis* chromosome appears to be heavily populated by segments of phage DNA sequences, with as many as seven integrated defective bacteriophage genomes per *E. faecalis* chromosome (29), relatively little is known about the infectious viruses of this species. Although phages capable of infecting strains of *E. faecalis* (formerly group D streptococci) have been known since 1922 (3), these and other *E. faecalis* viruses identified in several early reports (5, 7, 8, 13, 14, 21, 28, 33, 44) were not characterized, either morphologically or in terms of DNA content or composition. In these early studies the phages were merely used as tools to type *E. faecalis* strains and little attention was paid to the biology of the viruses themselves or to how they might interact with the host cell. In subsequent studies (1, 2, 20) the morphology of the *E. faecalis* phages identified by these investigators was described; however, as will be shown below, these phages differ substantially from the viruses that are the subject of the present investigation.

In this study, we describe the isolation and preliminary characterization of novel bacteriophages induced from root canal isolates of *E. faecalis*. To our knowledge, this is the first report of bacterial virus isolation from an infected root canal, and the first evidence of lysogeny in bacterial root canal isolates.

Materials and methods

Isolation of *Enterococcus* strains

Clinical enterococcal strains were isolated from root canals of teeth undergoing endodontic retreatments. The sampling procedures were carried out as described by Möller (26). After rubber dam isolation, tooth surfaces were cleansed and disinfected with 5% iodine tincture and 30% H_2O_2 before access. The pulp chambers were then accessed and the bulk of the gutta-percha root canal filling was removed using manual and rotary instrumentation. Each root canal was flooded with VMG-I sampling buffer (26) and the canals were then further instrumented to maximize recovery of organisms from the canal walls. The VMG-I sampling buffer (and all suspended material) from each root canal was absorbed into sterile paper points, which were deposited into sterile vials containing 1 ml of the same buffer. After vortexing, each sample was streaked onto double strength thallium acetate agar (0.2% thallium acetate, 1% peptone, 1% yeast extract, 1% glucose, 1.3% agar, 0.01% triphenyl tetrazolium chloride) (22). After overnight incubation at 37°C, representative purple/red colonies were picked, Gram-stained, and inoculated into brain–heart infusion (BHI) broth adjusted to pH 9.6. Gram-positive, streptococcal isolates growing at pH 9.6 were frozen and stored as presumptive enterococci. The identity of the presumptive *E. faecalis* isolates was confirmed using the API 20 STREP identification kit (bioMérieux, Marcy l'Etoile, France) following the manufacturer's instructions. The following laboratory *E. faecalis* reference strains were also used in this study: MMH594 (resistant to 500 μg/ml gentamicin), JH2-2 and OG1RF (resistant to 25 μg/ml rifampicin and 10 μg/ml fusidic acid), kindly provided by Dr Nathan Shankar (University of Oklahoma Health Science Center, College of Pharmacy). Strain OG1SSp (Str^r 1000 μg/ml, Spc^r 250 μg/ml) was obtained from Dr Gary Dunny, University of Minnesota Department of Microbiology.

Phage induction

Phage induction procedures were carried out based upon methods described previously (23, 39). Overnight BHI broth cultures of 10 confirmed *E. faecalis* strains (designated TUSoD 1, TUSoD 2, TUSoD 3, TUSoD 9, TUSoD 10, TUSoD 11, TUSoD 12, TUSoD 15, TUSoD 17, and TUSoD 18) were transferred as a 1% inoculum into 100 ml of BHI broth, kept at 37°C, and absorbance readings were made every hour, using a Spectronic® Genesys™ 5 (Spectronic Instruments, Inc, Rochester, NY) spectrophotometer at 600 nm. When the absorbance reached 0.2–0.3, mitomycin C was added to a final concentration of either 2.0 or 4.0 μg/ml. Growth was monitored for an additional 5 h, for a total of 7 h from the beginning of the experiment. Growth curves were produced for each strain. After the mitomycin C treatment, the cultures were centrifuged at 10,400 ***g*** for 10 min (GSA Rotor, Sorvall/Thermo Fisher Scientific, Asheville, NC) and the supernatants were rendered cell-free by filtration through sterile, 0.45-μm surfactant-free cellulose acetate membrane filters (Nalgene®; Rochester, NY). Phages present in the cell-free filtrates were sedimented by ultracentrifugation (186,000 ***g*** Beckman 45Ti rotor, Beckman Coulter, Inc., Fullerton, CA). The supernatants were discarded and the pellets were resuspended overnight in 300 μl 0.1 M ammonium acetate buffer at 4°C. The suspensions were kept on ice until processed for electron microscopic observation.

Electron microscopy

A sample of each concentrated phage suspension was prepared for electron microscopy using the following protocol. A drop of each sample was applied to a formvar-coated copper grid and after 2 min the liquid was withdrawn with a Kimwipe. The sample was then negatively stained for 30 s with either 1% phosphotungstic acid, pH 7.4, or a saturated aqueous solution of uranyl acetate. After removing the staining solution, as above, the samples were examined in a transmission electron microscope (JEOL 1200 EX II, Jeol Ltd., Tokyo, Japan) at ×40,000 to ×100,000 magnifications and 60 KV.

Host range screening of induced phage

A second induction experiment, using 4 μg/ml mitomycin C, was performed with *E. faecalis* strains TUSoD 1, TUSoD 2, TUSoD 3, and TUSoD 11. Following centrifugation in the 45 Ti rotor at 186,000 ***g***, the resulting phage pellets were resuspended overnight at 4°C in 500 μl SM buffer (50 mM Tris–HCl, pH 7.5, 100 mM sodium chloride, 8 mM magnesium sulfate, 0.01% gelatin), and each suspension was spotted onto separate lawns of all the confirmed *E. faecalis* root canal isolates and all of the five reference *E. faecalis* strains to determine their sensitivity to each of the four induced temperate phages. Following overnight incubation at 37°C, the presence of clear areas (zones of lysis) within the opaque overlay of each strain indicated sensitivity to the induced phage.

Purification of phage ϕEf11

A large-scale induction procedure was performed to obtain greater amounts of *E. faecalis* bacteriophage **ϕ**Ef11. The phage purification procedures were based upon methods described in Sambrook et al. (35) and Stevens et al. (41). Ten liters of BHI broth was inoculated with a 1% inoculum of an overnight culture of *E. faecalis* strain TUSoD 11. This culture was then incubated for 2 h at 37°C, until the optical density at 600 nm reached 0.2–0.3, whereupon mitomycin C was added to a concentration of 4 μg/ml, and incubation at 37°C was continued overnight. DNAse I was then added to a final concentration of 1 μg/ml and the lysate was held at room temperature for 30–60 min. The culture

was then clarified by low-speed centrifugation (GSA rotor at 10,400 ***g*** for 10 min and then 16,300 ***g*** for 5 min). The resulting supernatant was collected and run through a Centrasette tangential flow filtration system (Pall Life Sciences Corp., Exton, PA), and concentrated to approximately 250 ml retentate. The retentate was centrifuged through cesium chloride step gradients (δ = 1.35, 1.50 and 1.70) in SM buffer, in a Beckman SW41 rotor 106,000 ***g*** for 2 h. The opalescent blue band which formed at the δ = 1.35/1.50 interface was collected from each gradient and the bands from several gradients were pooled and dialysed overnight at 4°C against SM buffer. The phage in the dialysed material was then pelleted (Beckman SW41 rotor 153,000 ***g*** for 2 h) and resuspended overnight at 4°C in 200 µl TE buffer [10 mM Tris–HCl pH 7.4, 1 mM ethylenediaminetetraacetic acid, (EDTA) pH 8.0].

Preparation of phage DNA

The DNA was extracted from mitomycin C-induced phage ϕEf11 by the procedures described by Sambrook et al. (35). Briefly, the purified phage suspension was digested with proteinase K (50 µg/ml) in a solution of EDTA (20 mM, pH 8.0) and sodium dodecyl sulfate (0.5%). This mixture was then incubated at 65°C for 1 h whereupon the DNA was sequentially extracted with phenol, a 50 : 50 mixture of phenol : chloroform and finally chloroform. The extracted DNA was precipitated with ethanol (2 volumes) in the presence of sodium acetate (0.3 M, pH 5.2). The precipitated DNA was pelleted by centrifugation (13,000 ***g*** for 8 min), washed with cold 70% ethanol, dried using a Centrivap concentrator and then resuspended in TE buffer.

Restriction endonuclease analysis

The purified ϕEf11 DNA was digested with a series of restriction endonucleases. The enzymes tested included: *Apa*I, *Bbvc*I, *Bgl*I, *Bgl*II, *BstE*98, *BstE*II, *Cla*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Hpa*I, *Hha*I, *Nco*I, *Nde*I, *Not*I, *Nsi*I, *Pst*I, *Pvu*I, *Pvu*II, *Sac*I, *Sal*I, *Sfi*I, *Sma*I, *Sna*BI, *Spe*I, *Sph*I, *Stu*I, *Sty*I, *Vsp*I, *Sba*I, *Xho*I, *Xma*I, and *Xmn*I. After incubation with each restriction enzyme, the resulting DNA digest was analysed by agarose gel electrophoresis, using ethidium bromide staining.

Results

Presumptive *E. faecalis* strains were isolated from 18 of 78 root canals of teeth undergoing endodontic retreatment. Of these 18 strains, 10 were confirmed with >99% probability as *E. faecalis* by API STREP identification. The other presumptive *E. faecalis* strains could only be confirmed as *E. faecalis* to a much lower probability (e.g. 55%), and these were not used further in this study. Of the 10 confirmed *E. faecalis* strains isolated from persistently-infected root canals, at least four (TUSoD 1, TUSoD 2, TUSoD 3, and TUSoD 11) were lysogenic, yielding inducible bacteriophages following treatment with mitomycin C. Spot testing of the lysates of these four lysogenic strains revealed differences in host range among these phages (Fig. 1, Table 1). Phage in the lysate of strain TUSoD 1 (phage ϕEf1) produced zones of lysis only when spotted onto lawns of *E. faecalis* MMH594. Phages in lysates of *E. faecalis* TUSoD 3 and TUSoD 11 both produced lytic zones in lawns of indicator strains TUSoD 18 and laboratory *E. faecalis* strain JH2-2. In addition, a clear zone was seen when the TUSoD 11 lysate was spotted onto a TUSoD 1 lawn. Finally, phage ϕEf2, in the TUSoD 2 lysate, appeared to have the widest host range among the phages tested, producing zones of lysis in indicator lawns of *E. faecalis* TUSoD 1, TUSoD 10, TUSoD 18, MMH 594 and JH2-2.

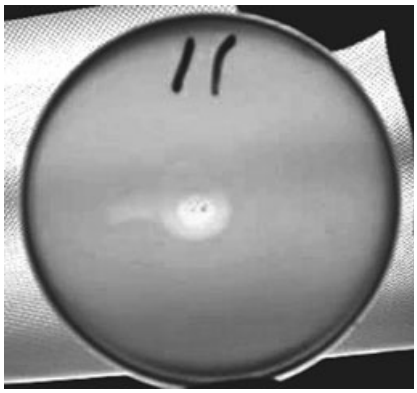

Fig. 1. Spot test showing a turbid lytic zone in lawn of indicator *Enterococcus faecalis* strain produced by a cell-free lysate of mitomycin C-treated lysogenic *E. faecalis* strain.

Electron microscopic observations revealed that the lysates of TUSoD 1, TUSoD 2, TUSoD 3, and TUSoD 11, all of which produced lysis zones in one or more indicator lawns, contained visible phage particles; these phages were designated ϕEf1, ϕEf2, ϕEf3, and ϕEf11, respectively. No phages were seen in the mitomycin C-treated cultures of any other *E. faecalis* strains. Three of the induced phages, ϕEf2, ϕEf3, and ϕEf11, had a morphology consistent with bacteriophages of the Siphoviridae family (18), which have small, spherical/icosohedral heads and long, thin non-contractile tails (Fig. 2B–D). The heads of these phages are approximately 41 nm in diameter and their tails are approximately 130 nm in length. In contrast, the morphology of phage particles seen in the TUSoD 1 lysate (ϕEf1) place them in the Myoviridae family, which have thicker, contractile tails (Fig. 2A) (18).

Incubation of phage ϕEF11 DNA with several restriction endonucleases (e.g. *BstE*98 and *Not*I) yielded no detectable digestion products. Conversely, many of the enzymes tested (e.g. *Eco*RI, *Hin*dIII, *Hha*I, *Xmn*I, and *Vsp*I) generated so many restriction fragments that they could not be used to estimate the size of the phage DNA. However, two of the enzymes tested, *Nde*I and *Nsi*I, generated distinctive restriction fragment patterns which could

Table 1. Host range for induced *Enterlococcus faecalis* bacteriophages

Indicator strains	Lysates from induced *E. faecalis* strains/phage			
	TUSoD1/ϕEf1	TUSoD2/ϕEf2	TUSoD3/ϕEf3	TUSoD11/ϕEf11
TUSoD 1	−	+	−	+
TUSoD 2	−	−	−	−
TUSoD 3	−	−	−	−
TUSoD 9	−	−	−	−
TUSoD 10	−	+	−	−
TUSoD 11	−	−	−	−
TUSoD 12	−	−	−	−
TUSoD 15	−	−	−	−
TUSoD 17	−	−	−	−
TUSoD 18	−	+	+	+
MMH594	+	+	−	−
JH2-2	−	+	+	+
OG1RF	−	−	−	−
OG1SSp	−	−	−	−
DG-16	−	−	−	−

+, clear, lytic zone observed in lawn of indicator strain; −, no clear lytic zone observed in lawn of indicator strain.

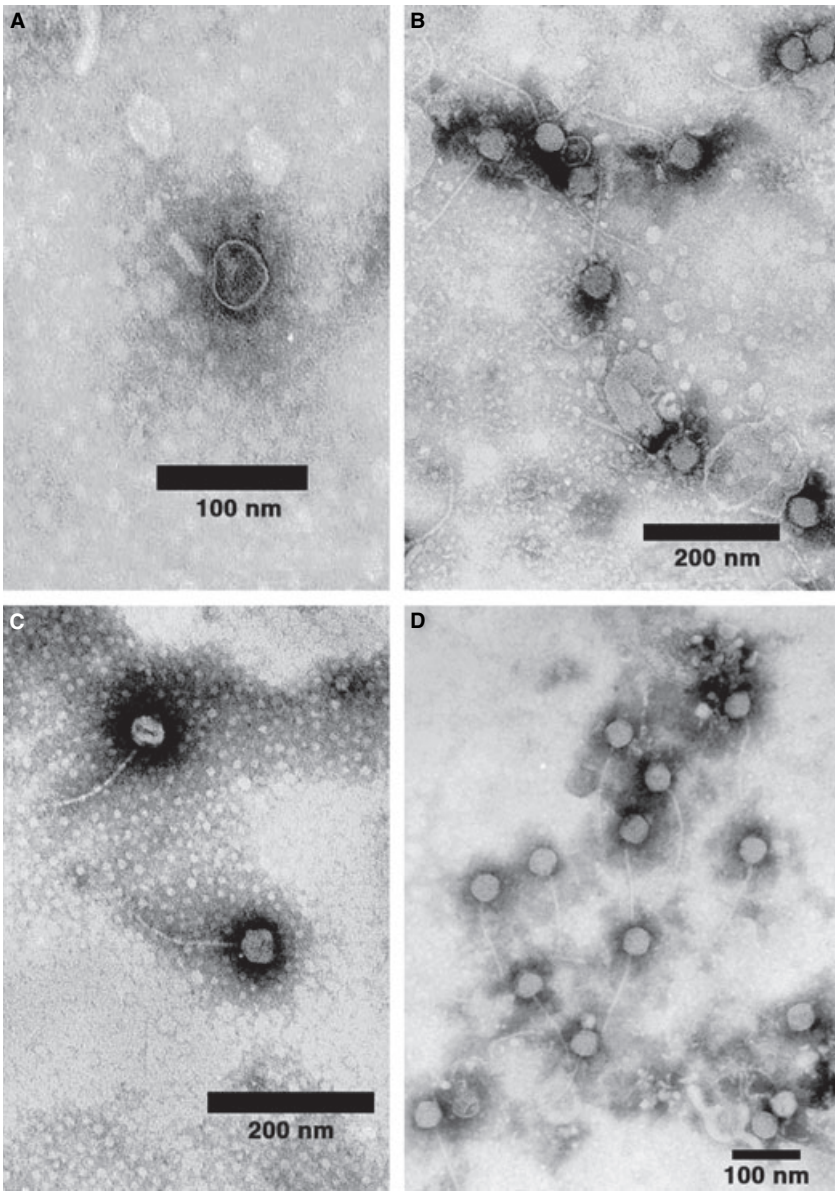

Fig. 2. Transmission electron micrographs of bacteriophages induced from *Enterococcus faecalis* root canal isolates. (A) Phage ϕEf1, induced from *E. faecalis* TUSoD 1; (B) phage ϕEf2, induced from *E. faecalis* TUSoD 2; (C) phage ϕEf3, induced from *E. faecalis* TUSoD 3; and (D) phage ϕ Ef11, induced from *E. faecalis* TUSoD 11.

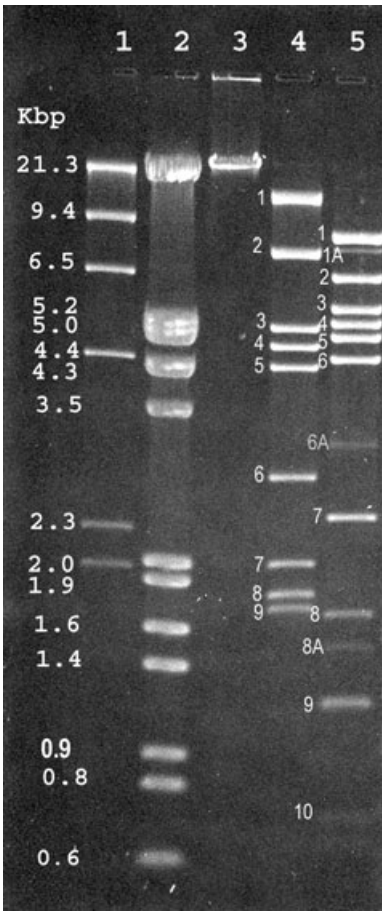

Fig. 3. Restriction endonuclease analysis of bacteriophage ϕEf11 DNA. Phage DNA restriction fragments were electrophoresed through a 0.7% agarose gel and stained with ethidium bromide. Lane 1, *Hin*dIII-digested λ DNA size markers; lane 2, *Eco*RI + *Hin*dIII-digested λ DNA size markers; lane 3, intact/undigested ϕ Ef11 DNA; lane 4, *Nde*I-digested ϕEf11 DNA; lane 5, *Nsi*I-digested ϕEf11 DNA. Sizes (in kilobase pairs) are indicated on the left.

Table 2. Size of phage ϕEf11 DNA restriction fragments

	Restriction enzyme	
Fragment	*Nde*I (kbp)	*Nsi*I (kbp)
1	11.0	8.3
2	7.5	6.6
3	5.3	5.7
4	4.8	5.3
5	4.3	5.0
6	2.8	4.5
7	2.05	2.4
8	1.85	1.65
9	1.75	1.30
10	–	0.7
Total	Σ41.35	Σ41.50

be used to estimate the size of the phage genome (Fig. 3). *Nde*I digestion of **ϕ**Ef11 DNA yielded nine restriction fragments ranging in size from 1.75 to 11.0 kbp. The *Nsi*I restriction pattern consisted of 13 restriction fragments, including three (1A, 6A, and 8A) that stained with ethidium bromide less intensely than other *Nsi*I fragments of smaller size (Fig. 3). This appearance was consistent throughout numerous gel analyses of the *Nsi*I restriction products of the **ϕ**Ef11 DNA; suggesting that these three DNA fragments were present in submolar amounts. This is probably linked to a circularly permuted genome as a consequence of headful packaging of concatemeric phage DNA during viral maturation. Based on the sum of the sizes of all the restriction fragments produced by each of the restriction enzymes, the size of the phage **ϕ**Ef11 genome can be estimated to be between 41.35 kbp (*Nde*I fragment total) and 41.50 kbp (*Nsi*I fragment total) (Table 2).

Discussion

Our recovery of *E. faecalis* from at least 10 (and possibly 18) of 78 root canals of teeth requiring endodontic retreatment is in agreement with numerous previous reports (12, 25, 31, 32, 42, 43) which indicate that *E. faecalis* is a common inhabitant of root canals of teeth with persistent periapical disease following root canal therapy. The

role, if any, of this organism in the persistence of disease remains to be determined; however, it is clear from previous studies that this organism survives many of the environmental challenges it encounters during the course of root canal treatment (17, 19, 37, 40).

To our knowledge, the present study is the first to report the isolation of lysogenic strains of bacteria from infected root canals. Furthermore, we recovered temperate phages from 4 of 10 *E. faecalis* strains examined. Although no statistical analysis can be made with such a small sample size, it would appear that lysogeny among endodontic *E. faecalis* strains is not a rare occurrence. Lysogeny among our endodontic *E. faecalis* isolates should come as no surprise because previous studies have documented lysogeny in salivary isolates of *E. faecalis* (28) and the sequencing of the genome of a vancomycin-resistant *E. faecalis* strain (V538) has disclosed the presence of seven phage regions within the chromosome (29). The significance of prophages in the *E. faecalis* genome remains to be explored. Although expression of most prophage genes is normally repressed, it is well known that in many cases, virulence-related genes are transcribed, resulting in modification of host properties such as toxinogenicity, antigenicity, and resistance to bactericidal activities (4, 47). In the case of the vancomycin-resistant *E. faecalis* strain V538, the prophage segments integrated within the chromosome contain genes that are homologous to *Pb1A* and *Pb1B* of *Streptococcus mitis* phage SM1, the gene products of which are known to promote bacterium–platelet binding (29). Since bacterium–platelet binding plays a critical role in the pathogenesis of infectious endocarditis, these phage genes may contribute to this significant health problem. Whether prophage gene products modify any of the properties of the *E. faecalis* strains infecting root canals is not known.

Another unique aspect of our results is the finding that bacteriophages can be isolated from material recovered from infected root canals. To our knowledge, this is the first report demonstrating the isolation of a virus from an infected root canal. Furthermore, our search of the literature suggests that the phages that we isolated from *E. faecalis* strains TUSoD 2, TUSoD 3, and TUSoD 11 represent new, hitherto undescribed, viruses. Although other *E. faecalis* bacteriophages have been previously reported, they have either not been characterized or, if characterized, their properties are clearly different from those of the phages described in the present investigation. Ackermann et al. (1) isolated an *E. faecalis* phage (VD13) from urogenital secretions. This phage had a long non-contractile tail typical of a Siphoviridae virus; however, unlike the phages we recovered from root canals, phage VD13 had a large, prolate head (113 nm long and 43 nm wide) (Fig. 4). Other phages which propagate in *E. faecalis* strains have been described by Jarvis et al. (20) and these phages also differ from those described in the present study by virtue of their contractile tails, characteristic of the Myoviridae family (Fig. 4). The *E. faecalis* phage particles detected in saliva by Bachrach et al. (2) were spherical, lacking any tail structure (Fig. 4). Yoong et al. (49) reported using enterococcal bacteriophage ϕ1, which has a contractile tail, to prepare a lysin that was active against several genera of gram-positive cocci. This Myoviridae type phage is morphologically distinct from the Siphoviridae phages that we recovered from the root canal *E. faecalis* strains TUSoD 2, TUSoD 3, and TUSoD 11. Other studies (7, 28) have used *E. faecalis* bacteriophages to type strains of *E. faecalis*; however, these phages were not characterized either morphologically or in terms of their genome content. It therefore appears that the phages described in the present study represent new, hitherto undescribed, viruses of *E. faecalis*.

It is of interest to note that with our recovery of inducible bacterial viruses from endodontic *E. faecalis* isolates, all four major forms of life (bacteria, archaea, eukarya, and viruses) have now been detected in infected root canals. Bacteria have long been known to infect root canals and the adjacent dentin of teeth with necrotic pulps (24). Similarly, eukarya, in the form of yeasts, have been detected in infected root canals for more than 40 years (10, 48). More recently, representatives of the Archaea domain have been detected in root canals by using molecular detection methods (45, 46). The present study documents the presence of viruses, as well, in infected root canals. Therefore, it now

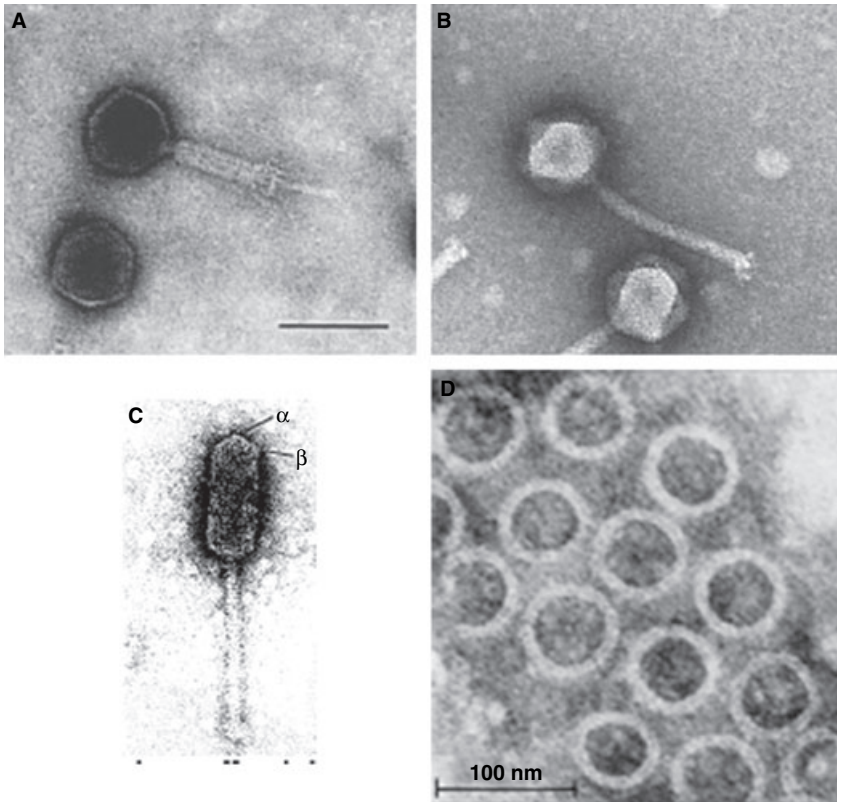

Fig. 4. Transmission electron micrographs of *Enterococcus faecalis* bacteriophages observed in previous studes. (A) *E. faecalis* phage 1 (note contractile tail), Jarvis et al. (20) (With kind permission from Springer Science+Business Media: Arch Virol. A study of five bacteriophages of the myoviridae family which replicate on different gram-positive bacterial. 1993: **133**: 75–84. Jarvis AW, Collins LJ, Ackermann H-W), (C) *E. faecalis* phage VD13 (note prolate head), Ackermann et al. (1) (reproduced with permission from publisher, NRC Research Press), (D) *E. faecalis* phage (note lack of tail structure), Bachrach et al. (2) (reproduced with permission from publisher, Wiley-Blackwell Publishing).

appears that endodontic infections may be more complex ecosystems than previously thought, consisting of any (or perhaps all) of the basic known life forms.

It has become evident, from recent studies using molecular techniques, that many organisms infecting root canals are not detected using cultural methods (27, 34). Similarly, viruses (or the proviruses of lysogenic bacteria), which are also not detected by conventional cultural techniques, have now been detected and isolated by using the methods presented in this study.

The significance of viruses in the endodontic infection ecosystem is not known. In other ecosystems, phages have been shown to modify the properties of the bacteria they infect and to be vehicles for horizontal transfer of genetic information between bacteria (6). Consequently, these viruses may influence the pathogenic potential of their host bacteria. Conversely, bacteriophages have been examined as possible agents to control their host bacteria. In this regard, it has recently been demonstrated that bacteriophages can significantly reduce, or even eliminate *E. faecalis* in infected root dentin *in vitro* (30). It remains to be determined how bacteriophages may impact endodontic infections clinically.

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