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Virology 329 (2004) 412–424

VIROLOGY

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Improved isolation of undersampled bacteriophages: finding of distant terminase genes

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Received 21 April 2004; returned to author for revision 16 August 2004; accepted 20 August 2004
Available online 25 September 2004

Abstract

Isolation and characterization of new environmental bacteriophages are performed for (1) analyzing microbial evolution and ecology and (2) delivering biological therapy. The sampling of environmental bacteriophages appears, however, to be limited by the procedure (usually liquid enrichment culture) used to propagate them. An alternative, less competitive procedure is developed here for the purpose of isolating new bacteriophages. This procedure involves extraction directly into and then propagation in a dilute agarose gel. Adaptations of this procedure are used to avoid repeated isolation of the same bacteriophage. Some newly isolated bacteriophages grow so poorly that they appear inaccessible to liquid enrichment culture. Four comparatively high titer bacteriophages were isolated and characterized by a genomic sequence survey. Some had genomes with extremely distant relationships to those of other bacteriophages, based on a tree built from the large terminase genes. These methods find novel genomes by rapidly isolating and screening diverse bacteriophages.

Keywords: Agarose; Bacteriophage 1102ϕ3-1; Environmental bacteria; Genomic nucleotide sequence; Nucleotide sequence homology

Introduction

Isolation and characterization of environmental bacteriophages are needed to assist analysis of microbial evolution and ecology, sometimes with the objective of antibacterial therapy. Evolution of bacteriophage genes is analyzed through nucleotide sequencing/informatics performed both for wild-type genomes (reviewed in Brussow and Hendrix, 2002; Campbell, 2003; Canchaya et al., 2003; Desplats and Krisch, 2003; Krylov et al., 2003) and genomes that are the product of controlled evolution (Bull et al., 1997; Hahn et al., 2002; Willats, 2002). Ecology of bacteriophages is analyzed through (1) either shotgun (Breitbart et al., 2002, 2003; Venter et al., 2004) or primer-directed (Zhong et al., 2002) nucleotide sequencing of unfractionated bacteriophage communities, and (2) isolating and characterizing of individual bacteriophages from the environment (examples are in Gill et al., 2003; Lu et al., 2003). Bacteriophage-based antibacterial therapy is usually performed with newly isolated environmental bacteriophages (Merril et al., 2003; Sulakvelidze and Morris, 2001; Summers, 2001; Stone, 2002).

To optimally use informatics in the above studies, a more complete global description of bacteriophage genomics is needed. Complete is meant in the sense that most genes in any new isolate are assignable to a previously known gene family. Ideally, at least one member of each gene family would have a known function. However, the current description of bacteriophage genomics does not yet come close to being complete, even ignoring the requirement for a homologue of known function. It is still true that newly reported bacteriophage genomes are replete with genes having no discernable homologues at all. Examples of recently reported bacteriophage genomes with many novel

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doi:10.1016/j.virol.2004.08.021

genes are the following: Pedulla et al. (2003) recently reviewed the genomics of 14 completely sequenced mycobacteriophages. The fraction of reading frames reported without identifiable homologues ranged smoothly from 6% to 76%. Rohwer et al. (2000) and Hardies et al. (2003) recently presented sequences of related bacteriophages isolated from the marine environment (Roseophage SIO1, Vibriophage VpV262); an average of 74% of reading frames had no identifiable homologues. Mesyanzhinov et al. (2001) recently reported the sequence of *Pseudomonas* bacteriophage ϕKZ; 81% of reading frames had no identifiable homologues.

Additional sequences are needed, first, to organize the currently novel genes into well-described families within superfamilies. Further, additional sequences are also needed to both bridge families and recognize even more distantly related homologues. The anticipated result is superfamilies that eventually have at least one member that is functionally characterized. Therefore, to become more complete, the current description of bacteriophage genomics must be augmented with data from additional bacteriophages that have previously undescribed genes.

As discussed in Hendrix (2002), the procedures generally used for the isolation and propagation of new bacteriophages appear to be highly biased and incapable of propagating most bacteriophage species. One reason for this conjecture is that environmental samples generally have a large physical abundance of bacteriophage particles while at the same time yielding few or no propagatable isolates (Hendrix, 2002; Paul et al., 2002; Rohwer, 2003; Wommack and Colwell, 2000). For example, the concentration in oceans, rivers, and lakes is usually 10^4–10^7 bacteriophage-like particles per ml and sometimes as high as 10^8 per ml (Fig. 3 in Chen et al., 2001), as judged by either electron or fluorescence microscopy (Chen et al., 2001; Lu et al., 2001). It was estimated that only a minor fraction (<1%; sometimes none) of the bacteriophage species represented have been detected by plaque assay. Recent investigations by electron microscopy reveal average bacteriophage concentrations of 1.5×10^8 per g in some soils (Ashelford et al., 2003; Williamson et al., 2003). None of these soil-borne bacteriophages visibly reproduced after extraction into liquid medium and inoculation into liquid bacterial cultures (liquid enrichment culture) in the case of Ashelford et al. (2003); attempts at culturing were not reported in Williamson et al. (2003). The sum of all efforts for 50 years is the isolation/propagation of only about 5100 bacteriophages total (Ackermann, 2001, 2003). Most (approximately 96%) are double-stranded DNA bacteriophages.

The typical procedure for isolating and propagating environmental bacteriophages is a liquid enrichment culture. Liquid enrichment culture is used for environmental samples that are either liquid (Breitbart et al., 2002; Lu et al., 2003; Sullivan et al., 2003) or solid (Ashelford et al., 2003; Breitbart et al., 2003). However, liquid enrichment culture may itself be a major limitation in the isolation of new bacteriophages for the following reasons. (1) In the case of bacteriophages on either a solid or semisolid environmental substrate (a biofilm in particular), the substrate has the potential for harboring low virulence bacteriophages that do not compete or even grow in liquid culture. (2) Even a more prolific bacteriophage growing in liquid enrichment culture must, in any case, compete against all other bacteriophages in the culture. Thus, the final product of a liquid enrichment culture may be only the bacteriophage most competitive in this culture. An illustration of this latter point is the isolation of bacteriophage T7. Bacteriophage T7 outgrew the other bacteriophages in a liquid culture that was supposed to be mixed (Summers, 1999). (3) Either bacteria or other organisms in the environmental sample may grow in a liquid enrichment culture and inhibit the growth of bacteriophages. The present study explores the use of an alternative procedure of bacteriophage isolation and propagation from a solid sample. This procedure is designed to reduce biological competition. New bacteriophages with unusual genomic DNA sequence are isolated and characterized.

Results

Primary culture: potential for isolation of more than one bacteriophage per sample

Primary cultures had a turbid lawn of host cells interrupted by (1) the soil sample and sometimes (2) zones of clearing. Some primary cultures had either one or a few well-defined zones of clearing. An example is in Fig. 1a. The host was *Bacillus pumilus*. The leftmost arrow in Fig. 1a designates clearing that appears to originate in one or more bacteriophage particles released from a soil particle near the center of the clearing. The rightmost arrow designates clearing that appears to originate in multiple bacteriophage particles.

Other primary cultures were more extreme. Some had no clearing (not shown). Some had more clearing than in Fig. 1a. For example, Fig. 2a shows a primary culture with numerous plaque-like zones of clearing (one is indicated by an arrow). The host is *Pseudomonas chlororaphis*. The plaque-like zones had borders less clearly defined than the borders of plaques of bacteriophages isolated from this primary culture (below). In addition to plaque-like clearing, background clearing was present throughout the culture of Fig. 2a. This background clearing is difficult to see in Fig. 2a, but is seen when the turbidity of most of the lawn is compared with the turbidity near the arrowhead in Fig. 2a. The primary culture was 16 h old in Fig. 2a. This culture completely cleared within the next 32 h at room temperature (not shown). Presumably, lytic bacteriophages cleared the culture.

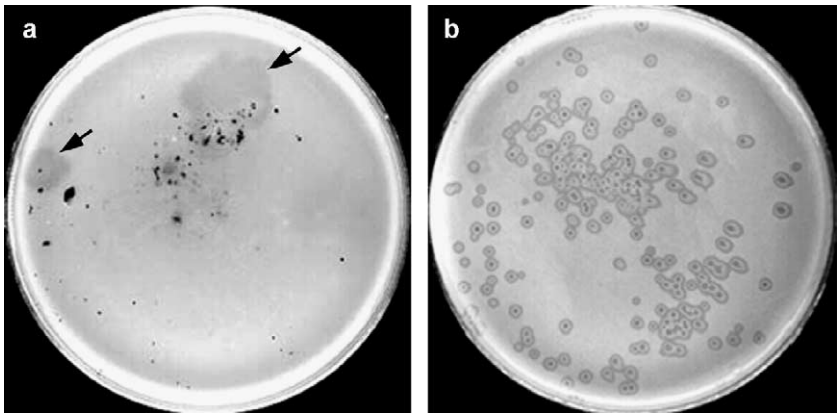

Fig. 1. Primary and secondary bacteriophage cultures with a *B. pumilus* host. (a) A primary culture (0.4% agarose gel) that appears to have both a single source (leftmost arrow) and multiple sources (rightmost arrow) of bacteriophages. (b) A secondary culture (0.2% agarose gel).

Secondary culture: screening bacteriophages

To minimize multiple isolations of the same bacteriophage, screening was performed during needle cloning. One screening criterion was plaque radius (R) vs. the concentration (A) of the agarose gel in which the plaques formed. This criterion was based on the assumption that sieving is the primary source of the gel's effect on R. In analogy with sieving during agarose gel electrophoresis (reviewed in Righetti and Gelfi, 1997; Serwer and Griess, 1999), this assumption implies the following: (1) The average R ($<R>$) decreases as A increases. (2) The dependence of $<R>$ on A becomes steeper as the bacteriophage particle being propagated becomes larger. A preliminary study confirms the accuracy of these assumptions in the case of bacteriophages G and T7 (Huang et al., 2001).

The above screen was applied by comparing plaque size in a 0.1% agarose gel to plaque size in a 0.3% agarose gel. One plaque former from the primary culture of Fig. 2a had an $<R>$ in a 0.1% gel (Fig. 2b) that was indistinguishable from the $<R>$ in a 0.3% gel (Fig. 2c). A second plaque former from the same primary culture had an $<R>$ in a 0.1% gel (Fig. 2d) that was visibly larger than the $<R>$ in a 0.3% gel (Fig. 2e). The plate pairs in Figs. 2b, c d, and e were incubated side-by-side. The bacteriophage of Figs. 2b, c (201ϕ2-2) should, by the above logic, be smaller than the bacteriophage of Figs. 2d, e (201ϕ2-1). Again, both of these bacteriophages were obtained from the primary culture of Fig. 2a.

Electron microscopy confirmed this conclusion. Figs. 2b, c had a tailed bacteriophage with a tail that was 130 nm long. The DNA-containing outer shell was 30 nm in radius (Fig. 3b). In contrast, Figs. 2d, e had a tailed bacteriophage with a tail that was longer, 210 nm. The outer shell was larger, 63 nm in radius (Fig. 3a).

Secondary culture: test of other procedures for screening

In addition to the two bacteriophages of Fig. 3, the primary culture of Fig. 2a also yielded four that were significantly and reproducibly different in plaque size/turbidity from both each other and the bacteriophages in Figs. 2b–e (0.2% agarose gel). But, all four were indistinguishable from both 201ϕ2-2 and each other by restriction endonuclease analysis (data not shown). No further tests were made of the differences among these latter bacteriophages. Thus, neither plaque turbidity nor plaque size (in a single gel) was a useful screen.

Secondary culture: unusual plaque type and additional hosts

Needle cloning also produced plaques of the following unusual, possibly new type from the primary culture of Fig. 1a: a clear center was surrounded by a turbid ring which was surrounded in turn by a clear ring; occasionally, another turbid/clear ring was observed (Fig. 1b; 201ϕ1-1 in Table 1). Progeny "bull's-eye" plaques were produced no matter where within a parental bull's-eye plaque a needle touched during cloning. Thus, the bull's-eye appearance is not caused by stable genetic variation of the bacteriophage within a plaque.

A second *B. pumilus* bacteriophage with bulls-eye plaques was observed after extraction/propagation from the surface of commercial Sudan grass (701ϕ1-2; Table 1). A third *B. pumilus* bacteriophage had a clear plaque phenotype after extraction from the surface of commercial oats (701ϕ1-1; Table 1). The Sudan grass and the oats had been stored dry for 5 weeks before use. Thus, these bacteriophages are resistant to drying when in the wild.

A *Bacillus subtilis* host was also used for primary and secondary cultures of soil. This host was further used in numerous attempts to isolate bacteriophages by liquid enrichment culture of the same soil samples. As previously found (Ashelford et al., 2003), the liquid enrichment cultures yielded no bacteriophages (data not shown). However, the procedure of Figs. 1 and 2 yielded a clear plaque-forming bacteriophage (1102ϕ3-1; Table 1).

Number of plaque formers per plaque: preparative growth

Preparative growth was initially tested in-gel, given that the bacteriophages were isolated by in-gel propagation. The

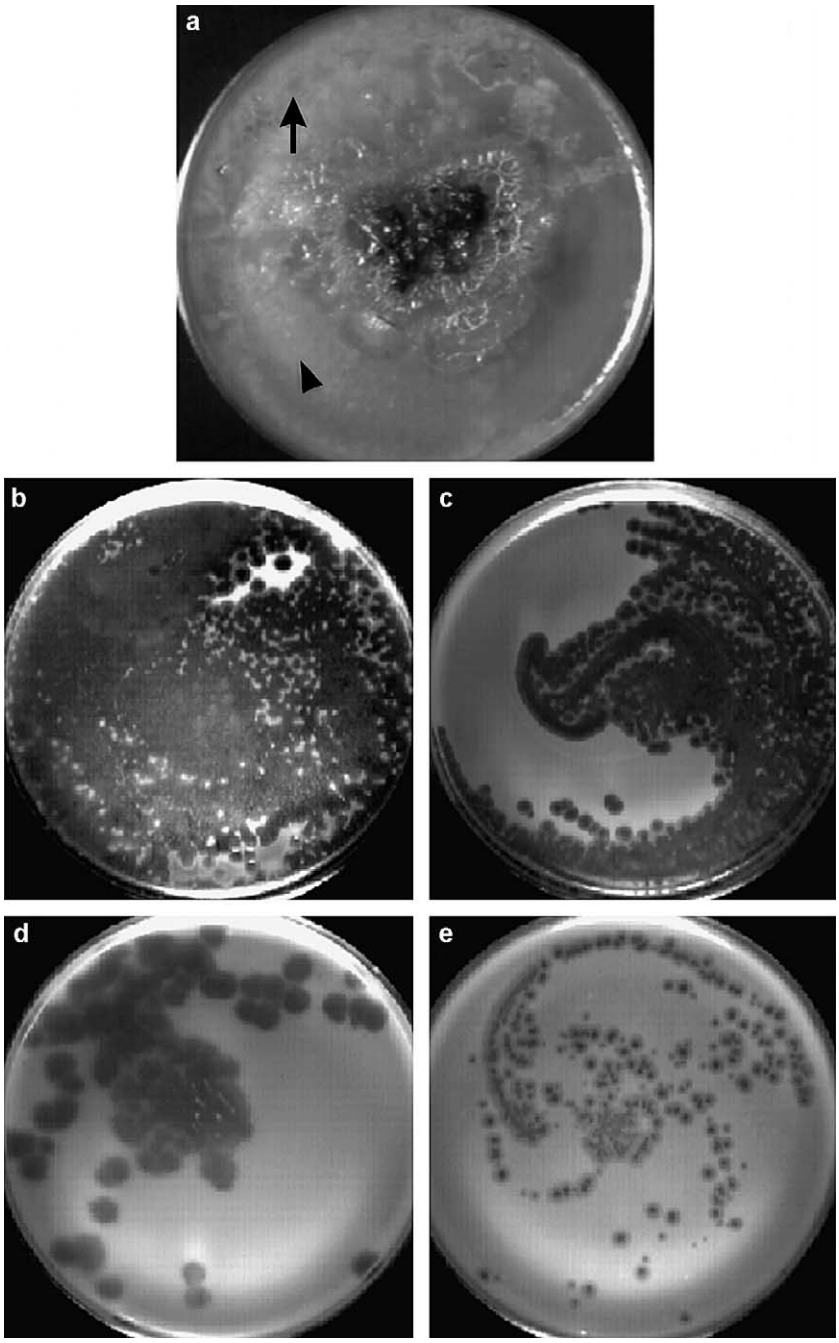

Fig. 2. Primary and secondary bacteriophage cultures with a *P. chlororaphis* host. (a) A primary culture (0.15% agarose gel) that has a comparatively high density of bacteriophage plaques with hazy edges. (b) A secondary culture of 201ϕ2-2 in a 0.1% agarose gel. (c) A secondary culture of 201ϕ2-2 in a 0.3% agarose gel. (d) A secondary culture of 201ϕ2-1 in a 0.1% agarose gel. (e) A secondary culture of 201ϕ2-1 in a 0.3% agarose gel. In (a), an arrow indicates a hazy-edged, plaque-like cleared zone; an arrowhead indicates a zone of bacterial lawn that appears to be comparatively uninfected.

PFU per plaque for 201ϕ2-1 and 201ϕ2-2 was surprisingly large ($>10^9$; Table 1). This result is more than an order of magnitude greater than the PFU per plaque of coliphage T7 (data not shown).

In contrast, the two bulls-eye bacteriophages produced less than 5×10^5 infective units per plaque (Table 1). This number is surprisingly low and prevented the use of a plate stock for preparative growth of the two bull's-eye bacteriophages.

Growth in liquid culture

For growth of 201ϕ2-1 in liquid culture, two plots of PFU/ml vs. time are in Fig. 4. The upper plot is for infection at a multiplicity of 1.0. The lower plot is for infection at a multiplicity of 0.001. The two plots did not differ significantly in shape. Both plots initially decreased. The titer at the minimum was about an order of magnitude lower

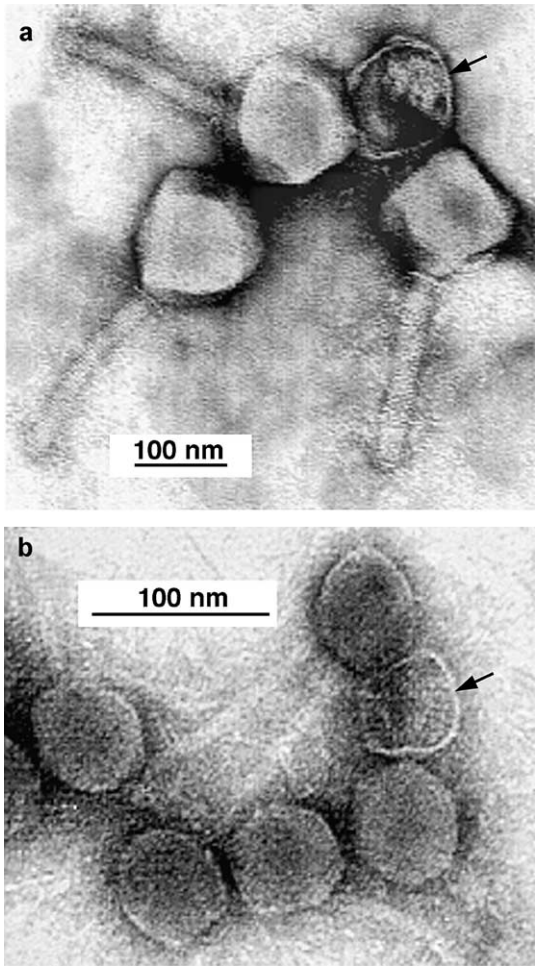

Fig. 3. Electron microscopy of 201ϕ2-1 and 201ϕ2-2. (a) 201ϕ2-1, (b) 201ϕ2-2. The arrows indicate capsids that appear not to have DNA.

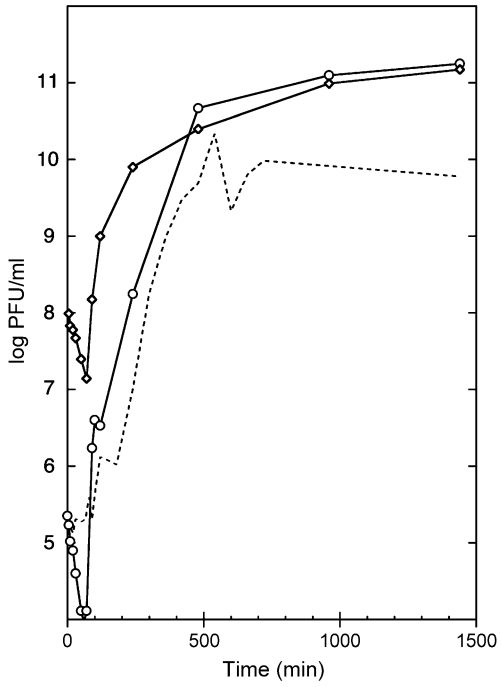

Fig. 4. PFU per ml vs. time. Results are shown for 201ϕ2-1 diluted into a diffusion-aerated culture of *P. chlororaphis* at two different multiplicities (1.0 and 0.001).

than the initial titer. After 1.5–2.0 h, the titer returned to its initial value. Then, the titer dramatically increased as a function of time. A plateau was observed at approximately 24 h. At the plateau, the plot usually achieved a titer close to 10^{11} per ml. The independence of plot shape from multiplicity indicates that the initial decrease in PFU/ml was not the result of several bacteriophages infecting the same cell.

To determine whether 201ϕ2-1 changed during its growth in Fig. 4, a lysate was used as the inoculum for a second PFU/ml vs. time plot. The result was a PFU/ml vs. time plot that did not significantly differ from the first plot (two trials; data not shown). Thus, the rapid growth phase of Fig. 4 is not caused by the selection of a rapid growth mutant.

The growth pattern in Fig. 4 was also found during liquid culture of coliphage T7 (dashed line in Fig. 4). Thus, this delayed growth pattern is not unique to the new bacteriophages. Interestingly, the peak titer for T7 was 2–3 × 10^{10} in two independent experiments. This titer is in the range of titers that one obtains with forced air aeration of a culture at either 30 or 37 °C (data not shown). This observation is not surprising when one considers that in the wild, cultures are not subjected to forced air aeration.

The experiment of Fig. 4 was repeated with 0.075% agarose added to growth medium. This agarose concentration was high enough for gelation (Serwer et al., 1988),

Table 1
Characteristics of newly isolated bacteriophages and hosts

Name[a]	Host names[b]	Growth: liquid[c]	Growth: in-gel[d]	Source
201ϕ2-1	*P. chlororaphis*: 200B-1	positive	1.2 × 10^{9}	soil
201ϕ2-2	*P. chlororaphis*: 200B-1	positive	1.4 × 10^{11}	soil
201ϕ1-1	*B. pumilus*: 301B-1	negative	1.8 × 10^{5}	soil
1102ϕ3-1	*B. subtilis*: 301B-5	positive	1.7 × 10^{8}	soil
701ϕ1-2	*B. pumilus*: *301B-1*	negative	2.9 × 10^{4}	oats
702ϕ1-1	*B. pumilus*: 301B-1	negative	1.4 × 10^{5}[e]	Sudan grass

[a] Official name as described in Materials and Methods.
[b] Host names as determined by the procedures described in Materials and Methods.
[c] Growth in liquid culture to a concentration of at least 10^{8} plaque-forming units per ml.
[d] Mean PFU per plaque. Standard deviations are ~0.5× the mean.
[e] In spite of the poor growth, a plate stock was made with enough particles to perform a DNA sequence survey.

but dilute enough so that the gelled agarose could be pipeted. The result was similar to the result without agarose, except that the initial drop in PFU per ml was approximately 5-fold, rather than 10-fold (data not shown). This reduction in the drop in titer was reproducible in side-by-side experiments and is significant. Perhaps, a more dramatic effect occurred in the more concentrated gels used for isolation and propagation without pipeting for infectivity assay.

The initial decrease in titer (Fig. 4) is puzzling, remembering that no separation was performed of host cell-attached from unattached bacteriophage particles. To test the possibility of killing by the elevated temperature of the plating block, the experiment of Fig. 4 was repeated with several platings of the sample taken at each time point. The first plating was made with the plating block at 60 °C; the others at 50 and 35 °C. The results did not significantly vary with the temperature of the plating block (data not shown). Assuming that temperature-caused inactivation would increase as temperature increases, the temperature of the plating block is not the cause of the initial decrease in titer. The cause is not known.

The experiment of Fig. 4 was repeated with both 201ϕ2-2 and 1102ϕ3-1. For 201ϕ2-2, the results were qualitatively the same as the result for 201ϕ2-1. For 1102ϕ3-1, the results differed in that the delay before increase in PFU/ml was shortened to 50–60 min (not shown).

The two bull's-eye bacteriophages grew slowly in liquid culture (aerated via either diffusion or forced air) to 10^4–10^5 PFU/ml when inoculated at a concentration of 10^3 per ml and incubated for 24 h (not shown). Thus, further investigation of the two bull's-eye bacteriophages was not conducted because of inadequate growth both in-gel and in liquid.

Genomic sequence survey: 201ϕ2-1

To screen for new genomic content, newly isolated bacteriophages were subjected to a sequence survey. Random clones from each genome were sequenced from the ends. Database searching then directly estimated the fraction of genes expected to be novel in each isolate. Survey results are shown for 201ϕ2-1, 201ϕ2-2, 1102ϕ3-1, and 702ϕ1-1 in Table 2.

For 201ϕ2-1, 16 of 22 sequence reads matched another bacteriophage gene, demonstrating that similarity can easily be detected when present in spite of the fragmentary nature of the survey data. This genome mostly had matches to reading frames from the previously mentioned one-of-a-kind bacteriophage ϕKZ genome. Most of these matches could not be extended into a larger gene family by Psi-Blast. Hence, although 201ϕ2-1 is not novel relative to ϕKZ, the two of them form a novel group within the viral kingdom. The fraction of genes estimated to be novel for 201ϕ2-1 (ignoring ϕKZ) is 86%, similar to 81% over the entire ϕKZ genome. Thus, bacteriophages within the ϕKZ group must evolve with only limited recombination with other known bacteriophages. As often found, the more mobile genes are nonstructural genes and include a self-mobilizing HNH nuclease.

A second measure of the novelty of the ϕKZ/201ϕ2-1 bacteriophage group is how long most of their genes

Table 2
Nucleotide sequence survey of new bacteriophage genomes[a]

201ϕ2-1 (22 survey reads total)	
iϕKZ orf 26	144/288, E = 1e−80
ϕKZ orf 27	90/152, E = 9e−49
ϕKZ orf 28	50/127, E = 3e−20
ϕKZ orf 29	45/66, E = 1e−18
ϕKZ orf 49	45/96, E = 2e−17
ϕKZ orf 50	16/36, E = 0.1
ϕKZ orf 70	60/134, E = 3e−26
ϕKZ orf 73	58/71, E = 2e−41
ϕKZ orf 74 (RNA polymerase)	156/214, E = 2e−81
ϕKZ orf 75	81/129, E = 9e−41
ϕKZ orf 92	18/47, E = 0.01
ϕKZ orf 96	51/182, E = 1e−14
ϕKZ orf 199	54/76, E = 9e−24
ϕKZ orf 198	41/109, E = 7e−13
NAD-dependent DNA ligase (various)	50/173, E = 4e−09
Lactobacillus phage phi-gle Rorf232 and related HNH endonuclease family	32/100, E = 7e−08
1102ϕ3-1 (20 survey reads total)	
NP_216884-phoH; phosphate starvation inducible protein	44/126; E = 1e−09
AAO93094-DNA polymerase from Bacteriophage Bastille	91/168; E = 2e−80
BAC65290-poly-gamma-glutamate hydrolase from Bacteriophage phiNIT1	110/131; E = 1e−55
201ϕ2-2 (15 survey reads total)	
NP_478191 and related HNH endionuclease family	26/61, E = 4e−06
NP_852777 from phage Aaphi23; distant homologue of P2 baseplate assembly protein gpJ	42/108, E = 3e−12
NP_852773 from phage Aaphi23; distant homologue of P2 baseplate assemby protein gpV	39/107, E = 6e−08
NP_775229 from phage PaP3; 5' to 3' exonuclease	73/208, E = 4e−17
702ϕ1-1 (14 survey reads total)	
NP_831664; large terminase from prophage phBC6A51	96/213, E = 2e−43
NP_831664; from prophage phBC6A51	44/120, E = 2e−13
NP_389619; NrdI homologue	27/58, E = 2e−05
NP_389620; NrdE (ribonucleoside diphosphate red.)	129/170, E = 8e−73
T13530; protein 41 of Bacillus phage phi-105	29/70, E = 4e−08
NP_657640; thy1	30/46, E = 3e−10

[a] Right and left boxes: Similarities as reported by Psi-Blast at http://www.ncbi.nlm.nih.gov/BLAST/ using the nr database as of January 2004.

have been isolated from exchange with genes of other bacteriophages. Among bacteriophage genes, generally the most conserved is the gene for an accessory protein/ATPase (called the large terminase) that participates in the packaging of bacteriophage DNA (reviewed in Catalano, 2000; Fujisawa and Morita, 1997). The large terminase gene of ϕKZ was not identified by the original authors (Mesyanzhinov et al., 2001), and is so diverged that it is unidentifiable either by either simple Blast searching, or reverse searching with CD search (Marchler-Bauer et al., 2003) with various terminase families found in pfam or COGS. However, a search using SAM found that the ϕKZ large terminase gene is ORF025 in GenBank.

An evolutionary tree is shown in Fig. 5 for the relationships of the various large terminase genes aligned using the SAM model. Timing is extraordinarily difficult to establish for evolutionary trees of bacteriophage genes. But, based on the discussion of host relationships in lambdoid phages in Desiere et al. (2001) and on the host relationships involved in the T7 series out to cyanobacteria (Chen and Lu, 2002), a gray zone in Fig. 5 is drawn to approximately indicate times up through the establishment of gram-positive and gram-negative bacteria. The ϕKZ branch plunges through the gray zone to an extremely ancient horizon. The ϕKZ branch occurs at a time before the times at which hierarchical relationships can be ascertained. Fig. 5 suggests that the ϕKZ large terminase gene, and by inference others of the ϕKZ genes, appear novel because their common ancestor with genes in other bacteriophages is ancient. Thus, hence homology is hard to detect.

Genomic sequence survey: the comparatively novel genome of bacteriophage 1102ϕ3-1

Blast searching for *B. subtilis* bacteriophage 1102ϕ3-1 survey sequences found homologues for only 3 nonstructural genes out of 20 reads. Among the three 1102ϕ3-1 genes that matched known genes, two were quite similar to the matched gene. This observation indicates a recent introduction into an otherwise alien genome. Hence, 1103ϕ2-1 appears to have an unusual genome and is currently being subjected to full genome sequencing. As with ϕKZ, either simple Blast searching or CD-search did not identify a large terminase gene, but searching with the SAM Hidden Markov Model did. On the large terminase tree (Fig. 5), the 1102ϕ3-1 branch also has ancient common ancestors with other bacteriophages. That relationship may be shared by others of its genes judging from the small percentage of genes with known homologues found in the survey.

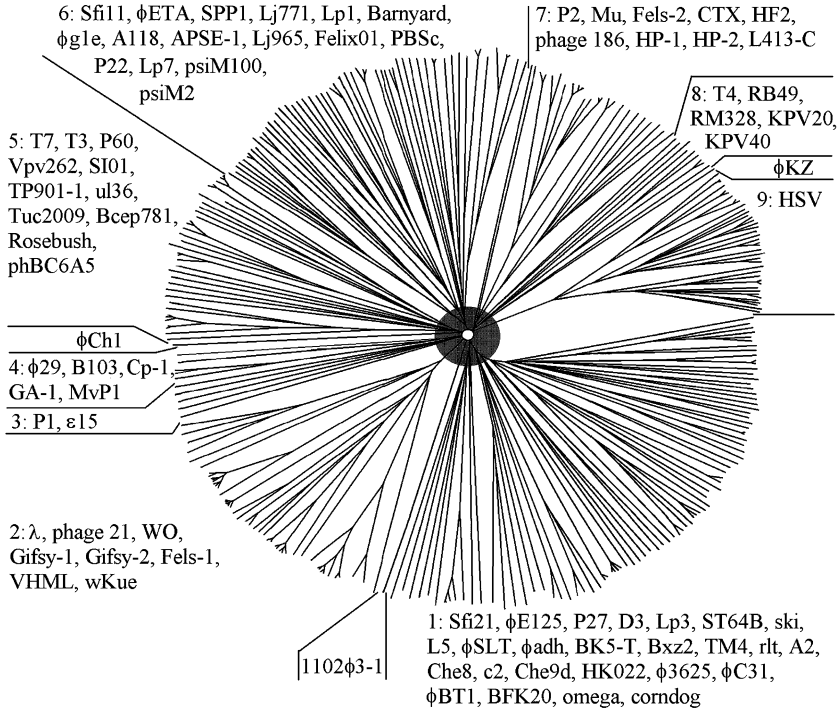

Fig. 5. An unrooted tree representing the relationships among large terminase genes. Results are shown for the following: tailed bacteriophages, prophages and herpes viruses. The extent of agreement with previous classification reflects the intensity of recombination among different types of bacteriophages. Around the margin, groups are presented that are based on bootstrap values above 80. Only the better known members of a group are listed. The most ancient position of the tree is the center (empty circle). Within the empty circle, nothing can be reliably concluded. The circle filled in grey represents subsequent times up to the time of the establishment of modern bacterial lineages (gram-positive/gram-negative/cyano: approximately 1.5 billion years). The time scale is greatly compressed in the grey circle. Three bacteriophage terminases stemming from the empty, innermost circle are marked: ϕKZ, 1102ϕ3-1 and ϕCh1.

Genomic sequence survey: the mosaic genomes of 201ϕ2-2 and 702ϕ1-1

For 201ϕ2-2, survey sequences included homologues to both structural genes and nonstructural genes. The structural genes in question most closely matched genes in bacteriophage Aaphi23, but more generally belonged to families of two of the genes in the P2 base plate assembly cassette. This cassette is scattered in some but not all members of several different large bacteriophage groups (P2, lambdoid, T4, and others not well classified). Both Aaphi23 and P2 are unrelated to 201ϕ2-1. Therefore, the conclusion is drawn that 201ϕ2-2 is also unrelated to 201ϕ2-1, even though 201ϕ2-2 and 201ϕ2-1 were co-isolated.

Similarly, *B. pumilus* bacteriophage 702ϕ1-1 was also found to have a mosaic structure. It matched at a higher portion of sequence reads (6/14) than the other isolates and its easily identifiable surveyed genes included part of the large terminase and an adjacent structural protein. The terminase closely matches phBC6A51, a prophage of *Bacillus cereus* (Ivanova et al., 2003) and the overall pattern of matches conforms to Hendrix et al. (2000) assembly from parts paradigm. Nonetheless, for both 201ϕ2-2 and 702ϕ1-1, Blast found that the reads not matching any homologue were greater than 50% of the total.

Discussion

New bacteriophages were isolated here by a procedure that was designed to separate a bacteriophage from interference that is either chemical (release of toxic substances from soil samples, for example) or biological. One can never be sure whether the bacteriophages isolated could ever have been isolated by liquid enrichment culture. However, the poor growth of the "bulls-eye" and 702ϕ1-1 bacteriophages makes extremely unlikely their isolation by liquid enrichment culture.

Furthermore, the kinetics found here for bacteriophage growth in liquid culture are not favorable to isolating more than one bacteriophage from a single sample. The bacteriophage that first gets to its rapid growth phase will dominate a liquid culture. In contrast, the in-gel procedures sometimes yield more than one bacteriophage in a single primary culture (for example, the primary culture in Fig. 2a). The extent to which potentially viable bacteriophages are missed by the in-gel procedures is not yet known.

Thus far, the most effective procedure for screening new bacteriophages is determination of the dependence of $<R>$ on A. Screening by the dependence of $<R>$ on A is based on the assumptions that bacteriophages are unrelated if and only if they differ in size. Although the first assumption holds in general, counter-examples do exist. One counter example is observed with P2 and P4 coliphages, related but different in size (Kim et al., 2001). In addition, bacteriophages indistinguishable in size are sometimes not closely related. Examples are coliphages P22 and T3/T7 (Casjens et al., 1992). Thus, screening by $<R>$ vs. A does introduce the risk of losing the opportunity to isolate some new bacteriophages. Unfortunately, use of either plaque turbidity or plaque size in a single gel was not found effective in further discriminating bacteriophages of similar size.

Survey results for bacteriophages 201ϕ2-1 and 1102ϕ3-1 suggest that after full sequencing they will have multiple genes defining new families and helping to bridge families together to superfamilies. Furthermore, 201ϕ2-1 in particular and possibly 1102ϕ3-1 appear to have been less exchange-prone and therefore capable of shedding light on the genome organization of ancestral forms. It would be unrealistic to imagine that any bacteriophage has descended billions of years without appreciable reorganization by recombination. However, targeting less exchange-prone genomes for sequencing can be expected to yield less obscured genomic fragments with which to attempt ancestral reconstructions. In contrast, bacteriophages 201ϕ2−2 and 702ϕ1-1 had genomes with mosaicism evident even at the survey level. Thus, the techniques used here produced bacteriophages of diverse levels of mosaicism.

The advance made here in culturing does not assure that all potentially viable bacteriophages are propagated. For example, plaques in primary culture have borders typically not as well defined as the borders of plaques in secondary culture. This observation suggests the presence of an event that causes at least a time delay in bacteriophage growth. This event might be as simple as desorption/diffusion of the bacteriophage from its dry residence. However, the effect of inhibitory agents is also possible. Knowledge of the members of the environmental bacteriophage population is further limited by the current failure to propagate most environmental bacteria (reviewed in Frohlich and Konig, 2000; Leadbetter, 2003; Palleroni, 1997).

Materials and methods

Media and buffers

Bacteria were grown in the following broth that was supplemented post-autoclaving with autoclaved 2.5% glucose: 10 g Bacto tryptone, 5 g KCl in 1000 ml water (growth medium). Growth medium was used in the lower agar layer of all Petri plates. Growth medium, supplemented post-autoclaving with 0.002 M autoclaved $CaCl_2$, was also used in gelled layers poured above the lower gelled layer. Bacteriophage suspension buffer was used for both purification of bacteriophages and storage of purified bacteriophages: 0.01 M Tris–HCl, pH 7.4, 0.01 M $MgCl_2$, 3% polyethylene glycol, molecular weight = 4000. The polyethylene glycol was added to the bacteriophage suspension buffer to stabilize bacteriophages (see Serwer et al., 1983). To inhibit bacterial growth in bacteriophage preparations

during storage, 0.01 M sodium azide was added to the bacteriophage suspension buffer.

Isolation, typing, and storage of bacteria

The bacteria used here were isolated from the environment by streaking for colony formation at room temperature (22 ± 3 °C) on a layer of pre-gelled 1.0% agar in growth medium. All bacterial strains were subjected to at least three consecutive single colony isolation/propagations before storage. Liquid cultures were aerated via diffusion. Storage of bacteria was achieved by, first, platinum needle transfer to a sterile solution of 10% dextran 10,000 in growth medium and then freezing at −70 °C.

To type bacteria, the following two procedures were used: [1] 16S rRNA gene segments were PCR amplified with the primers: 27F AGAGTTTGATCMTGGCTCAG, and 1492R GGYTACCTTGTTACGACTT, and sequenced with primers e27F and 519R GWATTACCGCGGCKGCTG, taken from Lane (1991). Data were analyzed using the Michigan State University Ribosomal Database Project website (Cole et al., 2003; http://rdp.cme.msu.edu/html/). [2] Gas liquid chromatography of methyl esters of the bacterial fatty acids was commercially performed (Microcheck, Inc., Northfield, VT). These two analyses of bacterial type were in agreement.

Isolation and storage of unpurified bacteriophages

To extract and propagate bacteriophages from a solid environmental sample (soil, for example), initially the environmental sample was placed in a Petri plate on the surface of a 1% agar gel cast in growth medium. Next, the following upper layer mixture was poured around the sample: molten agarose (4 ml, held at 50 °C) and overnight liquid culture of the host (approximately 0.1 ml, added from a dropper bottle). Most of the environmental sample was usually submerged. This upper layer mixture was made to form a gel by incubation at room temperature. The agarose for the upper layer gel was Seakem Gold agarose, BioWhittaker, Rockland, ME; the concentration of agarose is indicated in the text. The Petri plate was incubated for 16–20 h at room temperature unless otherwise indicated. The result was a "primary culture" that had a bacterial lawn that sometimes had zones of clearing. Examples of primary cultures are shown in Results.

Platinum needle transfer to a gelled lower agar layer was used to clone and propagate bacteriophages. To provide host cells for growth, an upper layer molten agarose–host mixture (4 ml molten agarose + 0.1 ml overnight host culture, at 50 °C) was poured on the bacteriophage-inoculated lower layer agar gel. To clone, the agar was inoculated only in the center; the molten agarose–host mixture was quickly swirled to non-uniformly spread bacteriophages. The upper layer was made to form a gel by incubation at room temperature. Incubation was continued at room temperature for 16–20 h to produce a "secondary culture". Usually, some regions of the secondary culture had confluent lysis of the bacterial lawn. However, other regions almost always had single plaques in a secondary culture, because the bacteriophages were spread non-uniformly. This procedure of single-plaque propagation will be called needle cloning.

Needle cloning of a bacteriophage was continued until the bacteriophage had experienced at least three consecutive single-plaque isolations. Then, a sample of a single plaque was stored by (1) platinum needle transfer to a sterile solution of 10% dextran 10,000 in growth medium, and (2) freezing at −70 °C. To document the plaques observed, either a digital or a conventional photograph of a Petri plate was obtained during epi-illumination with white light. Plates with dilute (0.075–0.2%) upper layer agarose gels were handled gently and were not inverted. Upper layer gels with agarose concentrations of 0.075–0.2% were solid enough to support plaques, but not solid enough to maintain integrity when a plate was inverted.

The bacteriophages described here had been isolated from dry samples and were, therefore, possibly drying-resistant. To test for bacteriophage contamination in the laboratory, plates with uninoculated bacterial lawns were incubated next to the plates with bacteriophage-inoculated bacterial lawns. These tests were always negative for bacteriophage contamination.

Naming of bacterial and bacteriophage strains

Bacteriophage strains are named by the month and year of isolation, followed by (1) the symbol, ϕ, (2) then, a number that designates the host, (3) next, a dash, and (4) finally, the number of the strain isolated in the month previously indicated. For example, 201ϕ3-4 is the complete name of the fourth bacteriophage strain isolated on host #3 in February of 2001. The names of bacteriophage strains used here are listed in Table 1.

Bacterial strains are given three names by necessity. The first name is the traditional name based on rDNA sequence and fatty acid composition. The second name is an analogue to the name given to bacteriophages, except that (1) the symbol, B, replaces the symbol, ϕ, and (2) the number immediately after the B is omitted. This second name is essential to be certain of the origin of any given strain because many strains will have the same traditional name. A third name (number) is needed to identify the bacterial strain when this strain is the host in the name of a bacteriophage. This third bacterial name is a simple number so that the bacteriophage names will not become too complex. This latter name has to be introduced because of the large number of both bacterial and bacteriophage strains whose isolation is anticipated. The names and numbers of the bacterial strains used here are in Table 1.

Preparative growth and purification of bacteriophage particles

For preparative in-gel growth of bacteriophages, the above procedure of needle cloning (0.15–0.2% upper layer agarose gel) was used with inoculation uniformly distributed around the lower layer agar gel. After incubation of the Petri plate, confluent lysis was typically observed for either all or most of the bacterial lawn.

After preparative in-gel growth of a bacteriophage, the following procedure was used to purify the progeny bacteriophage particles: first, the upper layer agarose gel was removed from a Petri plate. Bacteriophage particles in the pieces of upper layer gel were extracted with the broth used for the upper layer gel. Then, the extracted bacteriophage suspension was clarified by pelleting of both cellular debris and pieces of gel at 3000 rpm for 10 min in a JA-25.5 rotor used in a Beckman Avanti J-25 centrifuge at 4 °C. The pellet was twice extracted and clarified; the supernatants were pooled and then re-clarified ("plate stock").

For further purification, bacteriophages were then pelleted by centrifugation of the pooled supernatants at 21,000 rpm for 1.0 h in a JA-25.5 rotor at 4 °C. The pelleted bacteriophage particles were resuspended in bacteriophage suspension buffer. In the case of the bacteriophages used for electron microscopy, purification was completed by centrifugation in a cesium chloride step gradient (Serwer, 1976).

Monitoring of bacteriophage growth in liquid culture

To monitor the time course of bacteriophage growth in liquid culture, diluted bacteriophage particles were mixed with a bacterial culture in growth medium. The bacterial culture had 2×10^8 bacteria per ml as judged by counting in a Petroff–Hauser chamber. The multiplicity of infection (ratio of plaque-forming particles to bacterial cells) is indicated in figure legends. The infected culture was incubated at 22 °C. To mimic the environment, some cultures were aerated only by diffusion into a shallow culture that was 0.6–0.8 cm high in a dropper bottle. Others were aerated by use of forced air (a bubbler) when indicated in the text. Samples of a culture were diluted and then plated for plaque-forming units (PFU) at several times after the start of the infection. Semilogarithmic plots of PFU/ml vs. time were made to present the data. To compare results with results for a known bacteriophage, this experiment was performed with bacteriophage T7 and the host, *Escherichia coli* BB/1 (Serwer, 1976; Studier, 1969).

To determine the PFU in a plaque, a plaque-supporting piece of gel was removed from a Petri plate with the wide end of a sterile Pasteur pipette (approximate plaque volume = 0.1 ml). The piece of gel was placed in 1 ml bacteriophage suspension buffer with 0.01 M sodium azide. Bacteriophage particles were eluted with shaking at 4 °C for 8 h. The PFU/ml of eluted bacteriophages was then determined for 10 plaques; the average is reported.

Electron microscopy

After purification, bacteriophage particles were dialyzed against bacteriophage suspension buffer. The bacteriophage particles were negatively stained with 1% uranyl acetate and observed by electron microscopy (Serwer, 1976).

DNA sequence surveys: informatics

To characterize genomic content of bacteriophages isolated, sequence surveys were conducted on clones derived by fragmentation of the genomic DNA by either HincII digestion or sonication. GenBank accession numbers are: BH854219-BH854232 (201ϕ2-1; Table 1); BH854233-BH854241 (201ϕ2-2; Table 1); BH860902-BH860915 (1102ϕ3-1; Table 1), BH897847-BH897860 (702ϕ1-1; Table 1), and AY527292 (terminase for 1102ϕ3-1; Table 1). Each sequence was converted to a multifasta file containing all six translated reading frames corresponding to the sequence and then subjected to Psi-Blast (Altschul et al., 1997) search of a local copy of both GenBank and a custom bacteriophage-only library (for increased sensitivity). The sequences were also subjected to RPS-Blast (Marchler-Bauer et al., 2003).

Comprehensive details of construction and characterization of the large terminase alignment and tree will be further described elsewhere. In brief, the Sequence Alignment and Modeling System (SAM; Karplus et al., 1998) was obtained, installed locally and configured to search a nightly updated version of NCBI's nr database on a 44 node Beowulf computing array. The starting seed was a 41-member alignment from Pfam (PF03354) representing the largest of many terminase families present in Pfam (Bateman et al., 2002) at the time. The SAM search and align script (target99) was run iteratively in its default mode. Each iteration searched nr, expanded the family, recomputed the hidden Markov matrix (HMM) family model and produced the best alignment of the family. After each iteration the family was examined for addition of (1) previously identified terminase genes from other viral families which was assumed to indicate successful expansion and generalization of the family model, and (2) nonviral eukaryotic genes which was assumed to indicate noise.

To successfully expand the family without addition of noise, interspersion of either one or more target99 runs with the tuneup option (Hughey et al., 2000) was found to be necessary after each iteration. The purpose of the tuneup is to assure optimal convergence of both the alignment and the corresponding HMM during the HMM building process. Completion of the tuneup was monitored by inclusion of duplicate sequences and waiting for co-

convergence. The tuneup also greatly improved the subjectively judged appearance of the alignments, for example, by excluding gaps from predicted secondary structural elements.

The iterative family expansion converged (meaning no more family members were found in nr as it existed at the time) after about a total of 3 weeks of CPU time. The final alignment had 423 family members drawn from the genomes of bacteriophages, bacteria and herpes simplex viruses. No non-viral eukaryotic genes were present. One gene from each completely sequenced tailed bacteriophage was included. All biochemically/genetically defined terminase genes were correctly identified by the program. No genes with an alternative annotated function were found to be included, although many genes annotated as "hypothetical" or of "unknown function" were included. The last terminase genes found before convergence were from the bacteriophage ϕ29 family and these required special case measures (use of constraints) to fall into a mutually consistent alignment.

Validity of the alignment was judged by the consistency of alignment of known motifs (Mitchell et al., 2002) and by the consistency of alignment of predicted secondary structure elements (not shown). The most divergent segments of the alignment were on the order of 9% amino acid identity between different families. CLUSTALW was found to be ineffective at reprocessing the SAM alignment. Hence, the SAM alignment was used directly in all subsequent computations. In this iterative mode, SAM automatically expands or contracts domain boundaries at each iteration based on its finding of a defensible alignment in each segment of the sequence. The final alignment included essentially all domains of the bacteriophage lambda terminase except for the C terminal portal-binding domain.

A tree was made with PAUP (Swofford, 2002) by application of the Neighbor Joining algorithm (Saitou and Nei, 1987) with bootstrap values (Felsenstein, 1985). The tree was improved by exclusion of both incomplete genes and selected genes around the base of individual clades. Improvement was judged by coalescence of many small clades and individual sequences without cladistic assignment into a smaller number of large clades with bootstrap support >80. This strategy, which we call a "framework tree analysis", is designed to provide some statistically defensible structure within crowded trees (Hardies et al., 2000). The framework tree had 368 members. Most excluded members were incomplete genes. No explicit rooting was performed, although the tree had a radiative structure of nine major clades and several individual sequences around a central node. The central node presumably represents the earliest point. Correction for multiple hits has not yet been applied. The geometric center (see Fig. 5, below) should not be assumed to be a true radiation because the distances across the central node are indeterminately large.

Acknowledgments

The authors thank Gary A. Griess for help with data processing. The authors gratefully thank the Texas Coordinating Board (003659-0071-2001), the Welch Foundation (AQ-764), the Kleberg Foundation, and the National Institutes of Health (GM24365) for financial support.

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