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Sequence of the Genome of the Temperate, Serotype-Converting, *Pseudomonas aeruginosa* Bacteriophage D3

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Temperate bacteriophage D3, a member of the virus family *Siphoviridae*, is responsible for serotype conversion in its host, *Pseudomonas aeruginosa*. The complete sequence of the double-stranded DNA genome has been determined. The 56,426 bp contains 90 putative open reading frames (ORFs) and four genes specifying tRNAs. The latter are specific for methionine (AUG), glycine (GGA), asparagine (AAC), and threonine (ACA). The tRNAs may function in the translation of certain highly expressed proteins from this relatively AT-rich genome. D3 proteins which exhibited a high degree of sequence similarity to previously characterized phage proteins included the portal, major head, tail, and tail tape measure proteins, endolysin, integrase, helicase, and NinG. The layout of genes was reminiscent of lambdoid phages, with the exception of the placement of the endolysin gene, which parenthetically also lacked a cognate holin. The greatest sequence similarity was found in the morphogenesis genes to coliphages HK022 and HK97. Among the ORFs was discovered the gene encoding the fucosamine *O*-acetylase, which is in part responsible for the serotype conversion events.

Upon infection of sensitive cells, the genomes of temperate bacteriophages have two pathways open to them: development associated with cell lysis and the release of progeny (lytic response), or repression of lytic development usually associated with integration into the host chromosome and maintenance in a quiescent state (lysogenic response). The best studied of the temperate phages is bacteriophage lambda, which infects *Escherichia coli* strains. This phage is the archetype of a group of phylogenetically related viruses called the lambdoid phages. In many cases temperate phages also alter the phenotype of the lysogenized cells, resulting in the production of toxins or expression of surface components resulting in alterations to the cells' antigenicity. This phenomenon is called lysogenic conversion.

Bacteriophage D3 was obtained from a clinical isolate of *Pseudomonas aeruginosa* by Holloway et al. (35), who noted subsequently that lysogenization of host cells by phage D3 resulted in a change in the cells' serological properties (34). Kuzio and Kropinski showed that the lipopolysaccharide isolated from the lysogens [PAO(D3)] lacked receptor activity for this phage and that the O-antigenic polysaccharide side chains were chemically altered (41). Specifically, the hydroxyl group at position 4 of the D-fucosamine residues became acetylated, and the bonding between the trisaccharide repeats changed from α1→4 to β1→4. This results in the change of serotype from International Antigenic Typing Scheme O5 to O16/20 (42, 43). Simultaneously, the cells become both immune and resistant to superinfection by D3. Extrapolating from the work with *Salmonella* phage ε15 (6, 45, 46), I hypothesized that three phage gene products might be involved in the conversion: an inhibitor of the α-polymerase, a new β-polymerase, and a fucosamine *O*-acetylase. Extensive early attempts to clone the conversion genes failed.

Work by Cavenagh and Miller (12) demonstrated that phage D3 integrates at two distinct loci on the *P. aeruginosa* genome, and subsequent studies showed that it probably utilized the Campbell model of insertion (25). Induction of D3 prophage results in low-frequency-transducing lysates in which genes adjacent to the *attB* sites are transduced (12). Sharp and colleagues cloned the D3 *cos* sequences into a broad-host-range plasmid and demonstrated efficient transductional transfer between *P. aeruginosa* strains with wild-type D3 (60). The latter observation served as the basis for the development of a cosmid cloning system for *P. aeruginosa* (62).

Electron microscopic studies have shown that D3 is a member of the B1 (isometric head) subgroup of the family *Siphoviridae*, possessing a head with a diameter of 55 nm and a long flexible tail (7 by 113 nm) possessing six tail fibers with terminal knobs (26). The genome is double-stranded DNA of 56.4 kb (25) with 9-bp 3′-extended cohesive ends (61). The reported base composition is 50 mol% GC (49), which is unusually low relative to that (67 mol% GC) of its host, *P. aeruginosa*.

We have previously described the cloning and analysis of those regions involved in immunity (21, 22) and capsid morphogenesis (26). The immunity region (repressor gene [*c1*] and antirepressor [*cro*], together with the left operator-promoter [O_LP_L] and right operator-promoter [O_RP_R] complexes) from D3, is clearly homologous in structure and function to the immunity region of lambdoid phages (21, 22). Furthermore, in the case of both D3 and λ, the repressor mRNAs originating from the promoter for repressor maintenance (P_{RM}) lack the typical prokaryotic ribosome-binding sites (Shine-Dalgarno box), and the first three nucleotides of the message code for formylmethionine. Another point, which supports our contention that D3 is a lambdoid phage, was the observation that the capsid morphogenesis genes (portal-protease-major head protein) are laid out in the same orientation as in coliphage HK97. In addition, the portal and head proteins of these two phages show considerable sequence identity. Last, the D3 capsid protein undergoes proteolytic processing and cross-linking during head morphogenesis in a manner identical to that of the coliphage (26).

While exhibiting considerable similarities, these two phages differ in some significant ways. Their host range is restricted to either *E. coli* (λ) or *P. aeruginosa* (D3), making the generation

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of recombinant phages problematic. Coliphage λ DNA exhibits extensive segmented base composition, while melting temperature (T_m) analysis with D3 DNA indicates that this occurs to only a limited extent in D3 (A. Kropinski, unpublished results). The two phages are heteroimmune; that is, their repressors do not bind to the other's operator sequences. In the case of D3, the mRNA transcript from P_{RM} originates from within O_R3, rather than upstream of O_R3 as it does in λ. Analysis of 2 kb of D3 sequence data to the left of the O_LP_L complex failed to demonstrate open reading frames (ORFs) with homology to proteins in lambda (e.g., N) or its relatives. While the DNA of coliphage λ possesses 5′-extended termini, that of phage D3, along with coliphages HK97 and HK022, has 3′-extended cohesive ends. These are 9 bases in the case of D3 (61) and 10 bases in the case of the latter two coliphages (38). In addition, D3, unlike other lambdoid phages which have been sequenced, encodes four tRNAs (63). Last, the sole cellular receptor for coliphage λ is the LamB protein, while the receptor for D3 appears to be lipopolysaccharide (41).

To fully elucidate the relationship between D3 and other phages, particularly of the lambdoid group, I have completed sequencing the genome of D3. Analysis of the phage gene data indicates once gain that viral evolution is a far more complex issue than simple family relationships would explain.

MATERIALS AND METHODS

Cloning and sequencing. Specific D3 *Hin*dIII, *Sal*I, *Eco*RI, *Sph*I, *Pst*I, *Xba*I, *Bcl*I, and partial *Sau*3A fragments were cloned into pGEM3Zf(+) and grown in *E. coli* DH5α (F$^-$ ϕ80d*lacZ*ΔM15 *recA1 endA1 gyrA96 thi-1 hsdR17* (r_K^- m_K^+) *supE44 relA1 deoR* Δ(*lacZYA-argF*) *U169*] (Gibco/BRL). The cultures were routinely grown in Terrific broth (Difco Laboratories) at 37°C. Plasmid DNA was isolated by the alkaline lysis procedure with treatments with boiled RNase A and extractions with phenol-chloroform-isoamyl alcohol (27:24:1, vol/vol/vol) in the presence of silicone high-vacuum grease (Dow Corning) (52) prior to ethanol precipitation. DNA sequencing was carried out at the Institute for Molecular Biology and Biotechnology (McMaster University, Hamilton, Ontario, Canada) and the Guelph Molecular Supercenter (Guelph, Ontario, Canada), using the dideoxy-chain termination method (58) and Applied Biosystems automated fluorescence sequencers. The complete sequence was obtained through sequencing the termini of cloned fragments and primer walking on the clones. On two occasions, PCR was required to obtain intervening sequence for adjacent contigs.

Sequence assembly and analysis. The results of automated sequencing were collected, stripped of poor-quality data and vector sequences, and assembled into contigs using Seqman II (DNASTAR Inc.). ORFs were analyzed using ORF Finder at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) and WebGeneMark.HMM (http://genemark.biology.gatech.edu/GeneMark/whmm.cgi). In addition, the Find ORF feature of SeqEdit (DNASTAR) was used to manually scan the sequence for potential genes. A compendium of online tools (http://www.queensu.ca/micr/faculty/kropinski/online.html) was used for analysis of the putative genes. Proteins translated at ORF Finder or "translate tool" (http://www.expasy.ch/tools/dna.html) were scanned for homologs using BLASTP (2, 3) at http://www.ncbi.nlm.nih.gov; their masses and isoelectric points were determined online at ProtParam tools (http://www.expasy.ch/tools/protparam.html). Where homologs were identified, the sequences were compared using CLUSTAL W (70) at the EMBL-European Bioinformatics Institute (http://www2.ebi.ac.uk/clustalw/). In addition, Genestream's (Institute de Génétique Humaine) program ALIGN at its website (http://www2.igh.cnrs.fr/bin/align-guess.cgi) was used to compare two sequences. Proteins were also examined using PROSITE (4, 32) for conserved motifs at ExPASy-Swiss Institute of Bioinformatics homepage (http://www.expasy.ch/tools/scnpsit1.html). To predict transmembrane proteins, two online programs were used: TMPred (33) at European Molecular Biology network-Swiss node (http://www.ch.embnet.org/software/TMPRED form.html) and TMHMM (65) at the Center for Biological Sequence Analysis at the Technical University of Denmark (http://www.cbs.dtu.dk/services/TMHMM-1.0/).

For basic analysis of the DNA sequence, including restriction sites and motifs, DNAMAN (Lynnon BioSoft, Vaudreuil, Quebec, Canada) and Omiga (Oxford Molecular Group, Campbell, Calif.) were used. The DNA sequence was scanned for putative tRNA species using tRNAScan (18, 47) at its website (http://www.genetics.wustl.edu/eddy/tRNAscan-SE/) and FAStRNA (19) at http://bioweb.pasteur.fr/seqanal/interfaces/fastrna.html. Potential integration host factor (IHF)-binding sites were assessed using MacTargsearch (27) at SEQSCAN (http://www.bmb.psu.edu/seqscan/seqform1.htm), while potential transcriptional terminators (7, 8) were assessed using the Genetics Computer Group program terminator at Bionavigator (http://www.bionavigator.com).

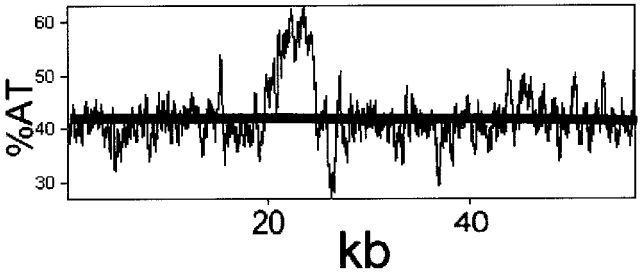

FIG. 1. Variation in AT content of D3 DNA presented as a function of genome length. The horizontal line corresponds to the average AT content (42.2 mol%), with the peak corresponding to the region associated with conversion.

Nucleotide sequence accession number. The phage D3 sequence has been deposited with GenBank (accession no. AF165214).

RESULTS AND DISCUSSION

Sequence of D3. We have previously published reports describing specific aspects of D3 and its sequence: analysis of the *cos* (61) and immunity regions (21), the existence of tRNA genes (63), and the identification of those proteins involved in DNA packaging and capsid morphogenesis (26). The complete nucleotide sequence of D3 is 56,426 bp, making it the largest *Pseudomonas* phage genome sequenced to date, the next largest being the 35.5-kb, cytotoxin-converting phage ϕCTX (53). This value agrees completely with the mass determined by restriction mapping (56.4 kb [25]) but is significantly less than values calculated from the sedimentation coefficient measured by Davison and colleagues (14) or the mass calculated from electron micrographs by Miller and colleagues (49). The work of Davison et al. (14) indicated that D3 DNA was significantly larger than the genome of phage F116, which Caruso and Shapiro have measured at 61.7 kb (10). Furthermore, a size of 65 kb can be calculated from the data based on electron micrographs and zone sedimentation by Miller and coworkers (49). While estimations of mass based on sedimentation would be influenced by the cohesive ends of the phage DNA which display a strong tendency to form hydrogen-bonded circles (62), the reason for the discrepancy based on electron microscopic data is unknown.

The overall base composition (57.8 mol% GC) is considerably higher than the published value (50 mol% GC [49]) but very similar to the value (58 mol% GC) derived on the basis of T_m analysis for the phage DNA (Kropinski, unpublished). This value is still significantly less than that of the host bacterium (67 mol% GC), which is unusual since the GC contents of temperate phage genomes usually closely match those of their hosts (Kropinski, unpublished). This may result in codon usage problems in *P. aeruginosa* (see below and reference 63). Unlike coliphage λ DNA, which displays segmented base composition (64), the T_m profile revealed only one region of differing base composition. This was verified by measuring variations in base composition across the genome length (Fig. 1). The only region of higher than expected AT content corresponds to the position of the serotype conversion gene (*orf28*), suggesting that this may have arisen by lateral gene transfer from another bacterium or phage with a lower GC content.

The restriction map published by Miller and Kokjohn (48) suggested multiple *Bam*HI sites, which I have been unable to verify. The map of Gertman et al. (25) also contains several errors of fragment order. The genetic map (Fig. 2) is presented in the same orientation as that of coliphage λ, with the repressor gene located to the right rather than the left, as published

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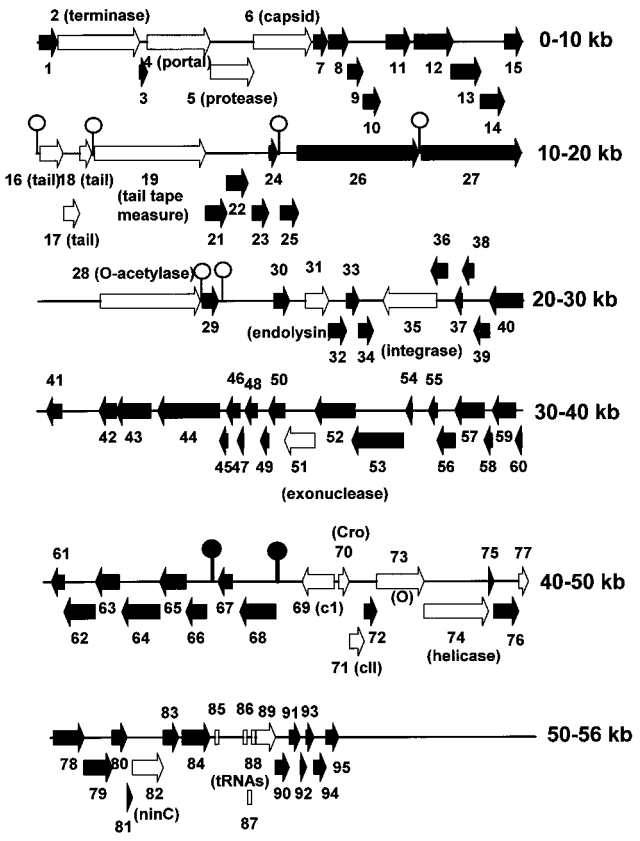

FIG. 2. Arrangement of ORFs in D3 DNA, indicating those which have significant sequence similarity or could otherwise be identified (open arrows) and those of unknown function (filled arrows). Potential Rho-independent terminators in the top and complementary strands are presented as open and filled lollipops, respectively.

previously (25). The previous *Hin*dIII map (25) indicated that the fragments were arrayed C-A-G-I-H-D-E-B-F; this has been corrected to C-G-I-A-H-D-E-B-F.

DNA motifs and their implication. A number of DNA motifs have been found in D3 DNA, including long direct repeats, Rho-independent terminators, and putative CII- and IHF-binding sites. Between nucleotides 29768 and 41830, seven direct repeats of 18, 23, 27, 28, 35, 210 and 247 bp were discovered. The significance of these repeats is unknown.

Lambda protein CII is an activator, stimulating transcription by binding to the opposite face of the DNA from that which the RNA polymerase binds in three promoters: P_{RE} (promoter for repressor establishment), P_I (integrase promoter), and P_{aQ} (anti-Q promoter). The P22 homolog, C1 protein, recognizes $TTGCN_6TTGY$ (30), while the lambda protein recognizes $TTGCN_6TTGC$ (31). A search for $TTGCN_6TTGY$ identified five sites in D3 DNA, two of which may play a role in development. A λ consensus CII-binding site ($TTGCN_6TTGC$), probably representing P_{RE}, was found within the *cII* gene (*orf71*). A site upstream of integrase (*int*) gene may represent a region homologous to the phage P22 promoter P_{aI}, which is a C1-activated anti-integrase promoter (30). Farinha and colleagues (21) identified a number of promoters, including P_L, P_R, and P_{RM} in D3 DNA through cloning into a chloramphenicol acetyltransferase reporter gene vector. They also reported that two strong promoters existed downstream of the immunity region which were not affected by repression in lysogens. One of these, contained within the *Sph*I R fragment, lies between bases 53879 and 54693, that is, between the tRNA genes and *orf90*, and may represent the late promoter for this phage. The fact that it is constitutive suggests that we have stripped it away from its normal regulatory circuits. While a number of potential σ54 promoters lie in this region, the exact location of the promoter and its function remain unresolved. This also applies to the promoter found in the *Sph*I B fragment (21).

In bacteria and their phages, termination of transcription occurs in a factor (Rho)-dependent (54) or -independent (13, 57) manner. The intrinsic mode of transcription termination is often characterized by a GC-rich sequence that could form a stem-loop structure immediately followed by a run of thymidine residues. Using the search algorithm of Brendel and colleagues (7, 8) I was able to identify many potential Rho-independent terminators. Those that fell between genes are listed in Table 1 and illustrated in Fig. 2. Interestingly, all of the putative terminators lie to the left of the repressor (*c1*) gene. Since one might expect that the morphogenesis genes would be expressed from the late promoter, the presence of sites following *orf15* and *orf18* suggests that these may function to modulate expression of downstream genes. The existence of factor-independent termination motifs flanking *orf29*, followed by a region apparently devoid of ORFs, suggests that the endolysin gene (*orf30*) may have its own promoter.

In many lambdoid phages, the switch from immediate-early to delayed-early gene expression is regulated through antitermination by a small (98- to 127-amino-acid) basic (pI 8.1 to 11.7) protein which in lambda is called N (24). In addition, antitermination is dependent on the presence of several host proteins, including RNA polymerase, NusA, -B, -E, and -G, Rho, and ribosomal protein S10, and *cis*-acting sites on the RNA. The Nut (N-utilization) region is comprised of two nucleotide motifs, *boxA*, which is conserved in sequence (CGCT CTTTAACA), and a downstream sequence (*boxB*) which is not (23). The latter is capable of forming a small stem-loop structure and is associated with RNA polymerase-NusA-NusG-S10 binding, while NusB and N bind to the former. We have detected a *boxA* site between genes 68 and 69, suggesting that transcriptional antitermination catalyzed by an N-type protein may exist in phage D3 early transcription (51) (Fig. 3). All attempts to identify N-like proteins using BLASTP or sequence examination for conserved motifs such as those found in P22, ES18, L (ICNIIDSIF), H-19B, HK97, or 933W (MTR RTQFKNSR) failed to identify an N homolog in D3. The possible exception is the product of *orf67*, which has a short motif (KARR) in common with ϕ21 gpN. A hyphenated stem-loop structure (GAGCCAACAGGCGC) is found 9 bp downstream of *boxA* and may function as the *boxB* analog in this phage.

TABLE 1. Putative Rho-independent terminators found in the D3 DNA sequence

Sequence[a]	Location
Direct	
CCCGCCTTGA**GCGGG**TTTTTTTGT	10019–10039
GCCCGGATCAT**TCCGGGC**TTTTTCATT	11255–11274
CCCGCTTCG**GCGGG**TTTTTTATT	15432–15451
CCCGCCATAGA**GCGGGC**TTTCTTAT	18597–18618
GCCCATCTAAATG**TGGGC**TTTATTTTAT	23373–23397
CGCCGGCCCTCGA**GCCGGCG**TTTTTGTT	24707–24728
Complement	
GCCCGCCTAGC**GCGGGC**TTTTTCAT	45047–45065
GCCCGGCAAGT**CCGGGC**ATTTTTT	43446–43466

[a] The region which might assume a stem-loop structure is bold-faced.

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FIG. 3. DNA sequence of the putative immediate-early leftward transcription region showing locations of the terminator downstream of *c1*, operators (O_{L1} and O_{L2}), and NutL (*boxA* sequence), each in boldface and underlined. Asterisks mark P_L, which overlaps the operators; the position of *orf68* is also indicated.

Description of selected ORFs. ORFs were designated very carefully, and as a result five regions apparently lack ORFs. These are between *orf27* and *-28*, *orf41* and *42*, *orf68* and *69*, and the first and second tRNA genes and from *orf95* to the end of the sequence. Otherwise, the ORFs were fairly densely packed, with many incidences of overlapping gene sequences. A total of 94 potential genes were discovered, of which 60 (63%) encoded polypeptides that showed no homology to proteins in the GenBank databases using the BLASTP search algorithm (Fig. 2; Table 2). The properties of some of those which showed sequence similarity will be discussed below, with special emphasis on the 17 ORFs that encode proteins that are significantly similar to proteins of characterized lambdoid phages.

Morphogenesis genes. We have previous shown that the pathway for capsid morphogenesis closely resembles that of coliphage HK97, with the prohead undergoing a number of transformations including proteolysis and cross-linking (26). Recent data from Juhala et al. on the completed sequence of lambdoid coliphages HK022 (GenBank accession no. AF069308) and HK97 (AF069529) proved most useful in the determination of which of the D3 gene products corresponded to other structural proteins (38). The sequence of ORF16 was 60% identical to that of HK022 gp12, which is the major tail protein. Interestingly, it is also shares 62% identity with a *P. aeruginosa* prophage protein. The product of D3 *orf17* is a protein of 119 amino acids that shares 56% identity with the 161-amino-acid-containing tail protein (gp13) of phage HK022. In addition, the products of D3 *orf10*, *-14*, and *-15* show 41, 42, and 58% identity with HK022 gp9, 10, and 11, respectively. Gene *19* specifies a 88-kDa protein with sequence similarity (32% identity) to tail tape measure protein of coliphage HK022.

Conversion. The 687-amino-acid product of *orf28* shows homology to a variety of proteins which have been identified as acetylases. These include the product of the *Salmonella enterica* serovar Typhimurium *oaf* gene and the WbpC protein of *P. aeruginosa* (GenBank accession no. 1545849; E [expect] value of 4×10^{-41}). In both cases, the overall sequence identity is 26%. Other hypothetical or inferred acetylases which show sequence relatedness include those from *Rhizobium* (1531614), *Mycobacterium* (CAA17305), *Bacillus*, and *Caenorhabitis* (2291126), with E values of 4×10^{-21} to 5×10^{-36}. This protein gave no hits with PROSITE but did so with pfam, indicating motifs found in a number of transport proteins including the xanthine/uracil permease family. Using TMPred and THMMH to evaluate this protein, 11 strong transmembrane regions are predicted with the amino terminus in the cytoplasm and the carboxy terminus in the periplasm. The original hypothesis had suggested that three proteins would be involved in the conversion event: an *O*-acetylase, a β-polymerase, and an inhibitor of the host α-polymerase (41). While the sequence evidence is strong for the first protein, neither of the others was identified in this study. In view of the fact that adjacent to *orf28* are regions apparently deficient in ORFs, it is possible that the other conversion genes map in these regions but are sufficiently dissimilar from the normal D3 ORFs to have been ignored. It is interesting that this protein has such a high molecular weight, while those acetyltransferases which modify antibiotics are significantly smaller (39). The multiple transmembrane domain of this protein may facilitate acetyl coenzyme A transport to periplasms or organization of a macromolecular membrane complex involved in O-antigen biosynthesis.

Lysis. The product of *orf31* is a polypeptide of 160 amino acids with strong homology to certain bacteriophage lyzozymes. The degrees of sequence identity to the λ, P2, 186, HK022, and HK97 endolysins are 54.5, 48.8, 48.2, 55.1, and 55.1%, respectively. Lambda endolysin, which has a high affinity for GlcNAc polymers (16), functions as a transglucosylase to cleave glycosidic bonds between the C-1 of MurNAc and C-4 of GlcNAc residues in cell wall peptidoglycan. Its active site has been defined by site-directed mutagenesis (37) as Glu19, which lies, with its side chain exposed, in a cleft between the two domains of the protein (20). In the case of the homologous protein in D3, which has a slightly longer amino-terminal region, this corresponds to Glu25. D3, HK97, HK022, and λ endolysins have two oligopeptides in common at the amino (AFLDMLAWSEGT) and carboxyl (CSNIWASLPG AGYGQ) termini of the proteins.

With the possible exception of coliphage T4 and *Staphylococcus aureus* phage 187, in all phages studied the endolysin gene is preceded or overlapped by a gene encoding a holin. This protein creates pores in the inner or cytoplasmic membrane permitting the endolysin to access the peptidoglycan layer in the periplasm, resulting in cell lysis and release of progeny viruses. In the case of T4, the holin gene is unlinked to the endolysin (E. Cutter, personal communication), while in phage 187 the holin gene is completely embedded within the endolysin gene (44). These proteins are characterized by their relatively small size (71 to 161 amino acid residues), the existence of two to three membrane-spanning helices, and poor sequence identity to other members of this group of functionally similar proteins (28, 72, 73). Furthermore, they sometimes possess a dual-translation start regulatory motif; for example, the holins of coliphages λ, HK97, and HK022 begin MetLysMet, whereas that of the phage 187 holin starts with MetLeuMet. With the possible exception of the *O*-acetylase, no D3 protein displaying these features was identified in the D3 sequence.

Integration. The sequence of integrase (ORF35) was 27.1% identical to the integrase sequence from filamentous *Shigella flexneri* phage SfX, which is a member of the virus family *Inoviridae*. It contained within it two conserved motifs, HDLRHT and RYAH. This gene and my analysis of the integration of the phage will be the subject of a future communication.

Immunity region. Our first published data on D3 (21) suggested that the immunity region was arranged very similarly to

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TABLE 2. Data on genes found in D3 DNA

Gene	Position		Initiation codon	Size of protein (kDa)	pI	Sequence similarity
	Left end	Right end				
1	37	417	ATG	14.1	8.7	
2	419	2110	ATG	63.3	5.2	**D3 terminase large subunit** *E. coli* hypothetical 50.9-kDa protein; 3e-77; P75978 (YMFN_ECOLI) Phage φ105 ORF22; 8e-43; BAA36628 Phage A2 ORF5; 4e-35; CAA66178 Phage HK97 terminase large subunit; e-33; AAF31098 Phage HK022 terminase large subunit; e-33; AAF30354 Phage φPVL ORF2; 2e-28; BAA31875
3	2107	2271	ATG	5.5	8.3	
4	2264	3568	GTG	47.3	8.8	**D3 portal protein** Phage HK022 portal protein; 6e-57; AAF30355 Phage HK97 gp3 portal protein; 3e-56; P49859 (VP3_BPHK7) Phage φC21 gp34; 7e-35; CAA07104
5	3572	4462	ATG	31.9	4.7	**D3 capsid protease** Phage φadh hypothetical protein; 3e-23; CAB52520 Phage φ7201 ORF25; 5e-18; AAF43518 Phage DT1 scaffolding protein; 6e-18; AAD21883 Phage Sfi21 *orf-221* Clp protease; e-15; AF115103
6	4459	5646	ATG	42.9	4.8	**D3 major capsid protein** Phage HK97 major capsid protein precursor GP5; 2e-69; P49861 (COAT_BPHK7)
7	5691	5978	ATG	10.2	9.7	
8	6005	6409	ATG	15.0	8.5	
9	6390	6710	ATG	11.6	4.2	
10	6711	7067	ATG	13.7	5.6	Phage HK022 gp9; 6e-11; AAF30361
11	7184	7684	ATG	18.9	8.8	Phage HK97 gp73; 6e-24; AAF31121
12	7763	8575	ATG	30.2	9.0	
13	8586	9143	ATG	21.2	5.6	
14	9136	9630	ATG	17.8	9.9	Phage HK022 gp10; 9e-21; AAF30362
15	9633	9998	ATG	13.3	4.9	Phage HK022 gp11; 7e-31; AAF30363
16	10074	10595	ATG	18.7	4.3	**D3 major tail protein** *Pseudomonas aeruginosa* prophage 28 (similar to V gene of lambda and *gene13* of N15) major tail protein; 1e-55; BAA83172 Phage HK022 gp12 (major tail protein); 8e-53; AAF30364
17	10605	10964	ATG	12.3	4.6	**D3 tail protein** Phage HK022 gp13 (lambda gpG analog); 2e-47; AAF30365
18	10970	11242	GTG	10.3	8.0	**D3 tail protein** Phage HK022 gp14 (lambda gpT analog); 1e-10; AAF30366
19	11292	13796	ATG	87.8	4.9	**D3 tail tape measure protein** Phage HK022 gp15 (tail length tape measure protein); e-82; AAF30367 Phage HK97 (tail length tape measure protein) gp16; 2e-25; AAF31092
21	13793	14272	ATG	18.4	5.4	
22	14256	14747	ATG	18.1	4.8	Phage MB78 hypothetical protein; 4e-04; CAB36891
23	14837	15208	ATG	13.2	4.5	
24	15219	15413	ATG	7.4	8.2	
25	15468	15875	ATG	14.9	8.4	
26	15847	18576	GTG	99.5	5.0	
27	18636	20879	ATG	80.3	5.3	
28	21282	23345	ATG	75.5	9.1	**D3 acetylase** *Salmonella* serotype Typhimurium O-antigen acetylase; 2e-53; U65941
29	23383	23715	ATG	12.5	8.8	
30	24857	25180	GTG	11.3	4.5	
31	25505	25987	ATG	17.3	9.0	**D3 endolysin** Phage HK97 lysin; 2e-41; AAF31145 Phage HK022 lysin; 2e-41; AAF31145 Phage lambda endolysin (gpR); 5e-41; P03706 (LYCV_LAMBD) Phage 186 P protein; 3e-32; AAC34155 Phage P2 lysozyme; 3e-32; P51771 (LYCV_BPP2)
32	25984	26352	GTG	12.6	6.9	*P. aeruginosa* prophage hypothetical protein; 1e-16; BAA83169
33	26349	26609	ATG	9.4	9.9	*P. aeruginosa* prophage hypothetical protein; 2e-14; BAA83170
34	26600	26908	ATG	11.4	6.3	
35	27104	28213C	ATG	41.4	9.8	**D3 integrase** Phage Sfx integrase; 8e-33; AAD10295 *E. coli* prophage DLP12 integrase; 4e-31; P24218 (INTD_ECOLI) Phage V integrase; 1e-30; AAB72135 Phage P22 integrase; 2e-30; CAA27685 Phage APSE-1 integrase; e-29; AAF03981 Phage SfII integrase; 2e-19; AAC39270
36	28099	28440C	ATG	12.5	8.9	
37	28588	28749C	ATG	6.1	3.8	
38	28746	28979C	ATG	8.5	6.1	
39	28979	29305C	ATG	11.8	6.6	
40	29302	29991C	ATG	25.8	4.9	

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TABLE 2—*Continued.*

Gene	Position		Initiation codon	Size of protein (kDa)	pI	Sequence similarity
	Left end	Right end				
41	30192	30503C	ATG	11.7	4.6	
42	31282	31641C	ATG	13.4	5.1	
43	31634	32356C	ATG	26.5	4.5	
44	32492	33772C	GTG	47.7	5.4	
45	33769	33942C	GTG	6.3	9.7	
46	33911	34195C	GTG	10.4	5.4	
47	34135	34266C	ATG	5.0	9.8	
48	34294	34551C	ATG	10.2	11.8	
49	34611	34787C	GTG	6.7	8.7	
50	34780	35115C	ATG	12.7	9.5	*P. aeruginosa* regulatory protein AlgR; 4e-10; P26275 (ALGR_PSEAE) *P. putida* PprA; 9e-10; CAA56559
51	35112	35738C	ATG	23.3	5.0	**D3 exonuclease** Phage VT-Sa exonuclease; 5e-06; BAA84296 Phage lambda exonuclease; 8e-06; P03697 (EXO_LAMBD) Phage 933W exonuclease; 8e-06; AAD25418
52	35742	36569C	ATG	29.9	6.7	
53	36503	37567C	ATG	40.2	5.4	
54	37615	37752C	ATG	4.8	10.3	
55	38086	38268C	ATG	6.6	10.2	
56	38265	38639C	ATG	14.1	5.1	
57	38636	39244C	ATG	22.8	5.1	
58	39237	39407C	ATG	6.5	9.1	
59	39410	39895C	ATG	18.1	5.6	
60	39880	40026C	GTG	5.5	4.5	
61	40161	40430C	ATG	10.0	5.7	
62	40423	41079C	ATG	25.0	5.5	
63	41083	41580C	GTG	19.1	8.6	
64	41630	42418C	ATG	29.5	4.5	
65	42415	42963C	ATG	20.6	4.6	
66	42960	43385C	ATG	15.6	4.5	
67	43628	43921C	ATG	11.1	8.8	
68	44080	44829C	ATG	27.4	7.7	
69	45369	46040C	ATG	24.6	5.6	**D3 C1 repressor** Phage D3112 repressor; e-14; S13498
70	46128	46349	ATG	7.9	7.9	**D3 Cro**
71	46351	46659	ATG	11.4	5.7	**D3 CII** Phage lambda CII protein; e-04; P03042 (RPC2_LAMBD) Phage 434 CII protein; 2e-04; P03043 (RPC2_BP434)
72	46659	46922	ATG	9.6	10.2	
73	46919	47908	ATG	37.0	8.9	**D3 DNA replication protein O** Phage HK97 gp54; e-06; AAF31132 Phage lambda replication protein O; 3e-05; AAA96584
74	47905	49245	ATG	48.9	5.4	**D3 DNA helicase** *Bacillus subtilis* helicase; e-75; P37469 Phage P1 Ban protein; e-69; CAA09719
75	49245	49364	ATG	4.6	7.9	
76	49361	49870	GTG	19.0	7.9	
77	49872	50075	ATG	7.8	4.5	Phage ϕCTX ORF39; 6e-09; AB008550
78	50065	50697	ATG	24.1	9.7	Phage APSE-1 P46; 4e-23; AAF03989 Phage ϕadh ORF188; 3e-16; CAB52497
79	50690	51277	ATG	22.1	6.6	
80	51274	51582	ATG	11.5	5.3	
81	51579	51692	ATG	4.1	5.7	
82	51689	52330	ATG	23.9	9.9	**D3 NinG** Phage P22 NinG; 2e-21; CAA55163 Phage lambda ORF204; e-20; P03770 (Y204_LAMBD) Phage 21 NinG; 2e-20; CAB39991 Phage 933W ORF15; 2e-15; CAB39297 Phage H-19B ORF204; 4e-15; AAD04653
83	52330	52647	ATG	11.8	9.5	
84	52715	53293	ATG	21.5	9.5	
85	53398	53473				Met-tRNA (CAU)
86	53980	54055				Gly-tRNA (UCC)
87	54068	54142				Asn-tRNA (GUU)
88	54147	54222				Thr-tRNA (UGU)
89	54241	54645	ATG	14.0	6.5	
90	54638	54922	ATG	10.5	10.0	Phage ϕCTX ORF10; 4e-6; AB008550
91	54922	55155	ATG	8.3	5.1	
92	55155	55280	ATG	4.6	10.0	
93	55273	55434	ATG	5.9	3.8	
94	55434	55685	ATG	9.4	9.7	
95	55682	55942	ATG	9.2	6.7	

[a] Emphasis is given to phage homologs. The similar protein is followed by the BLAST E value and the GenBank accession number (and in some cases sequence designation) of that protein. Proteins clearly identifiable by experimental or BLAST analysis are in boldface.

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that of coliphage λ. Furthermore, while sequence similarity between λ CI and D3 C1 proteins was poor, residues which were shown be to essential for λ repressor function were strongly conserved in the D3 repressor. The O_RP_R complex contained three operators and two promoters (P_R and P_{RM}) as it did in phage λ. One fundamental difference exists between the spatial arrangement of genes in the classical lambdoid phages and in *Pseudomonas* phage D3, that is, the lack of intervening nucleotide sequence between *cro* and *cII*. In λ this region contains a Rho-independent transcriptional terminator (t_{R1}) in addition to sites involved in antitermination.

The region to the left of the *c1* gene is illustrated in Fig. 3. This region contains two putative Rho-independent terminators (t_{L1} and t_{c1}), the Nut site, and two genes (*orf67* and *orf68*). Overlapped *orf67* is another potential genes (*orf67A*) which is not listed in Table 1 but contains a sequence (THW-P-PEPPQ) which is also found in two hypothetical proteins, the products of genes L0065 and *orf4* from coliphages 933W (GenBank accession no. AAD25410) (57) and VT2-Sa (BA84287) (50), respectively. This protein would have a mass of 6.9 kDa and a pI of 5.8. Furthermore, the alleged P_L overlaps the O_{L1} and O_{L2} and has the sequence **TTGACA**ACGAATATGAGCAAT CT**CATACT**. The bases in bold match the consensus σ70 promoter sequence in the −35 box completely and at the −10 site by four out of six bases.

DNA replication. We have shown that replication of D3 DNA involves a switch, as it does in λ, from a theta to a sigma mode during lytic development (60, 62). DNA replication in coliphage λ involves assembly of an activated replication complex involving gpO, gpP, and a variety of host proteins, including primase (DnaG) and helicase (DnaB), at the origin of replication *ori*λ (69). This region also contains DnaA-binding sites (68). While D3 DNA encodes a 37-kDa basic protein with homology to λ gpO, there is no indication of a gpP homolog or DnaA-binding sites (consensus TTWTNCACA [59]). In its stead D3 possesses, as do certain other phages including *Salmonella* serovar Typhimurium phage P22 (36), a helicase homolog with a mass of 49 kDa. In this case, the greatest sequence similarity is shown to the *Bacillus subtilis* DnaB helicase.

tRNAs. Using tRNAscan-SE (47) and FAStRNA (19), Sibbald and Kropinski identified four tRNA genes in the delayed-early region of the bacteriophage D3 genome (63). These are specific for methionine (AUG), glycine (GGA), asparagine (AAC), and threonine (ACA). In D3, Thr- and Gly-tRNAs recognize codons which are rarely used in *P. aeruginosa* and presumably influence the rate of translation of phage proteins. Two codons, AGA (Arg) and AUA (Ile), are rarely used in *E. coli* but employed more frequently in coliphage λ. It has been noted that the λ integrase has a higher proportion of the rare arginine codons AGA and AGG and that this influences expression of this gene (74). Taking two pairs of proteins which one would expect to be expressed at different levels, capsid (*orf6*) and major tail protein (*orf16*), compared with repressor (*orf69*) and integrase (*orf35*), it was noted that certain codons are favored in the highly expressed genes. These include UUC (Phe) and AAC (Asn), while UCA (Ser), AUA (Ile), ACA (Ala), AGG (Arg), and both GGA and GGG (Gly) are selected against in the highly expressed proteins. This presents a conundrum as to why this phage should have tRNA genes for Gly_{GGA}, Asn_{AAC}, and Met_{ATG}.

Phage evolution. The phylogeny of phages has been discussed in two excellent reviews by Campbell (9) and Casjens et al. (11). Relationships have been hypothesized through similarities in morphology, conservation of gene arrangement, ability to recombine, cross-hybridization patterns, and sequence. Hendrix and colleagues have stated that while conserved patterns exist, which indicates familial relationships, the overall picture suggests that considerable intervirus or virus-host recombination has occurred, often between distant bacterial groups (29). Their proposition is that all double-stranded DNA phage genomes are "mosaics with access, by horizontal exchange, to a large common genetic pool but in which access to the gene pool is not uniform for all phages." The data presented for D3 show clear evidence that this type of evolutionary process may have operated in the evolution of this *Pseudomonas* phage, making it the first bacteriophage outside the family *Enterobacteriaceae* that clearly shows phylogenetic relatedness to members of the lambdoid family of coliphages. With minor exceptions, including the placement of the lyzozyme gene, the genomic layout, particularly the morphogenesis and immunity-replication regions, mimics that of lambdoid phages. This is borne out by the sequence data which suggest that D3 is most closely related to HK022 and HK97, both well-recognized members of the lambdoid group. In part, this may be expected since the databases are overrepresented by data from enterobacterial phages and have only a limited selection of genome data from the large *Pseudomonas* phages. While *E. coli* and its relatives and the fluorescent pseudomads (55) are both members of the γ subdivision of the phylum *Proteobacteria* (66), they are only distantly related. Other intriguing data suggest a relationship between D3 and members of the *Siphoviridae* infecting gram-positive bacteria, particularly those of the lactic acid bacteria of the genera *Streptococcus* and *Lactobacillus*. D3 proteins involved in packaging (terminase large subunit) show sequence similarity to analogous proteins of *B. subtilis* phage ϕ105 (K. Kobayashi, K. Okamura, T. Inouse, T. Sato, and Y. Kobayashi, unpublished data [GenBank accession no. BAA36628]) and *Lactobacillus casei* phage A2 (J. E. Suarez, unpublished data [GenBank accession no. LCA251790]), while the capsid maturation protease appears related to ClpP proteases from *Lactobacillus gasseri* phage ϕadh (1) and *Streptococcus thermophilus* phages ϕ7201 (67), DT1 (71), and Sfi21 (15). Furthermore, in the case of many of the phage genomes for gram-positive bacteria, the endolysin genes are found downstream of those involved in morphogenesis whereas in the classical lambdoid phages these genes are located before those genes involve in morphogenesis. These results suggest a bipartite ancestry to D3 involving recombination between phages of gram-positive bacteria and a protolambdoid phage with the packaging originating among the phage of gram-positive bacteria, while the remainder of the phage genome evolved from lambdoid phages of gram-negative bacteria. This could occur following the superinfection of a common host cell by two different species of phages (or DNA) or through recombination between superinfecting and resident prophage genomes. Those phages that had the ability to infect different species could then pass on the new genomic segments, ultimately resulting in unrelated bacteriophages possessing homologous genes. From an ecological perspective, lactic acid bacteria and pseudomonads have been isolated from the rumen (5), while both *Bacillus* species and pseudomonads are soil microorganisms, resulting in the potential for genetic exchange through transformation (17, 40).

In all phages examined to date, a considerable percentage of the ORFs do not encode proteins with homologs in the current database. It is imperative that GenBank contain more annotated phage sequence data, representing complete phage genomes, so that useful conclusions can drawn about the nature of these unknown genes and the evolution of phages.

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