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Rapid Communication

Genomes of "phiKMV-like viruses" of *Pseudomonas aeruginosa* contain localized single-strand interruptions

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ABSTRACT

The "phiKMV-like viruses" comprise an important genus of T7 related phages infecting *Pseudomonas aeruginosa*. The genomes of these bacteriophages have localized single-strand interruptions (nicks), a distinguishing genomic trait previously thought to be unique for T5 related coliphages. Analysis of this feature in the newly sequenced phage φkF77 shows all four nicks to be localized on the non-coding DNA strand. They are present with high frequencies within the phage population and are introduced into the phage DNA at late stages of the lytic cycle. The general consensus sequence in the nicks (5′-CGACxxxxxCCTAoh pCTCCGG-3′) was shown to be common among all phiKMV-related phages.

Introduction

The T7-related *Podoviridae* include well characterized lytic bacteriophages infecting various bacterial genera (Dunn and Studier, 1983; Garcia et al., 2003), and are grouped within the proposed *Autographivirinae* subfamily, as they encode their own RNA polymerase and have a conserved genome organization (Lavigne et al., 2008). The "phiKMV-like viruses" comprise a distinct genus within this subfamily, and seem to be environmentally important as they appear ubiquitous and infect a wide range of *P. aeruginosa* strains (Ceyssens et al., 2006). Genomes of several phages, including phiKMV (Lavigne et al., 2003), LKD16 (Ceyssens et al., 2006) and LUZ19 (NC_010326) have been extensively analyzed. Differences between this genus and the "T7-like viruses" include the late localization of the phiKMV RNA polymerase and the lack of conserved (T7-like) promoter elements for this polymerase (Lavigne et al., 2003; Ceyssens et al., 2006).

In this manuscript, the genome sequence of bacteriophage φkF77, isolated in Russia in 1983 and a close relative of phiKMV, is presented. Previous studies conducted on φkF77 suggested the presence of canonical (localized) nicks within its DNA (Kulakov et al., 1985), a feature previously only known for T5 and its relative BF23 (Abelson and Thomas, 1966; Shaw et al., 1979; Wang et al., 2005). The T5 genome contains five major (canonical) nicks, present in 80 to 90% of its DNA molecules and at least six minor nicks that are present with a frequency of 5 to 10% (Jonston et al., 1977). All nicks are located in one of the DNA strands (Abelson and Thomas, 1966; Hayward and Smith, 1972). No function has been attributed to these nicks, although viable T5 mutants have been obtained lacking major/minor nicks as well as mutants with increased frequency of canonical nicks (Rogers et al., 1979; Rhoades, 1984). Here we demonstrate that the presence of localized single-strand interruptions with a conserved 14 nucleotide sequence is a distinguishing feature of the genome organization of phiKMV and its relatives.

Results and discussion

Initial indications of the presence of single-strand interruptions (nicks) within phage genomes were obtained from the results of the agarose gel electrophoresis of φkF77, phiKMV, LUZ19 and LKD16 DNA denatured by alkali. This produced distinct bands for all analyzed phages (Fig. 1, odd numbered lanes). The interruptions were readily repaired by treatment of the DNA preparations with T4 DNA ligase (Fig 1, even numbered lanes), showing they contain adjacent 3′-OH and 5′-phosphate groups. The nicks in φkF77 were visualized by electron microscopy and their occurrence in the phage DNA estimated. In Fig. 2A, a partially denatured DNA molecule of φkF77 is presented; four major nicks (α to δ) can be seen as denatured regions with the characteristic free single stranded ends. Eighty molecules were analyzed, and all four canonical nicks were localized in the φkF77 genome (Fig. 2B).

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It is known that in phage T5, all nicks are located in one of the DNA strands (Abelson and Thomas, 1966; Hayward and Smith, 1972). To determine if *P. aeruginosa* phages have the same structure, the φkF77 ssDNA fragments (Fig. 1) were individually isolated and hybridized to each other. The products of hybridization were treated with SI nuclease. Only when fragments II, III, IV, V or VI were hybridized with fragment I (numbering in Fig. 1) the double stranded DNA products were detected by gel electrophoresis (results not shown).

The ssDNA fragments produced as a result of φkF77 DNA denaturation were separately analyzed as described previously (Nichols and Donelson, 1977b; Ksenzenko et al., 1987). The 5′-ends of ssDNA fragments IV, V and VI (numbering as in Fig. 1) have an identical six nucleotide sequence -pCTCCGG, while the common 3′-end sequence of each was determined as -CCTA$_{OH}$. Sanger sequencing of the non-coding strand of φkF77 led to a full stop of the DNA polymerase at the predicted nick sites. Pretreatment of the DNA preparations with T4 DNA ligase was sufficient to completely restore sequencing reactions (Supplementary Fig. S1).

Whole-genome sequencing of the φkF77 genome and alignment verified (and expanded; see below) this motif, with locations corresponding to those determined by electron microscopy experiments (Table 1, Fig. 2). Although both approaches agree qualitatively, there is a quantitative discrepancy between the sequencing and electron microscopy data. For example, sequencing essentially stopped at nick δ (Fig. S1), suggesting that the template was completely nicked, but only 33% of the molecules were scored as being nicked by microscopy (Fig. 2B). However, sequences flanking nick δ have the highest apparent T_m (followed in reducing order by γ, α and β). It is likely that electron microscopy of partial denatured DNA underestimates the nick frequency. It is important to note that frequencies estimated from sequencing data are also likely to be inaccurate. We suggest that nicks are present in a majority of molecules at all sites, and at site δ almost all molecules are nicked.

To investigate at which stage of the lytic cycle the localized nicks are introduced, intracellular DNA isolated from *P. aeruginosa* PAO1 infected with φkF77 was analyzed by primer extension (Supplementary Fig. S2). Localized nick α is repaired soon after infection but reappears after approximately 20 min of infection, at the same time as infectious phage particles become detectable. In control experiments, phage DNA was detected on all stages of infection using phage-specific pair of primers (LK1 and LK3; results not shown). These results indicate that localized nicks are apparently absent in the replicating phage DNA and are most likely to be introduced at the time of packaging.

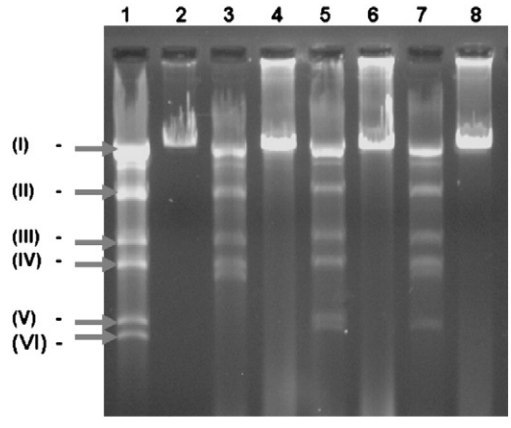

Fig. 1. Identification of the localized nicks in DNA molecules of phiKMV group bacteriophages. DNA preparations from bacteriophages φkF77 (lane 1), phiKMV (lane 3), LUZ19 (lane 5) and LKD16 (lane 7) were denatured in 0.1 M NaOH and subjected to electrophoresis in 0.9% agarose gel. Electrophoresis patterns of the same DNA preparation treated with T4 DNA ligase prior to denaturing in 0.1 M NaOH are presented in lanes 2, 4, 6 and 8. Single-strand DNA fragments of φkF77 are numbered as I, II, III, IV, V and VI.

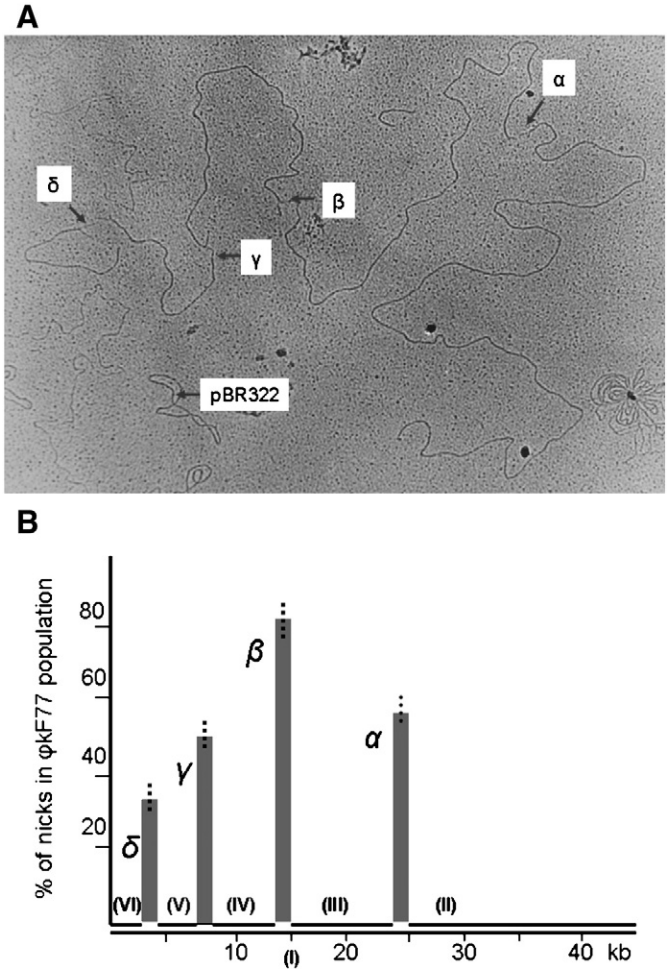

Fig. 2. Localisation and analysis of the distribution of nicks in bacteriophage φkF77. (A) Electron micrograph of a partially denatured DNA molecule of φkF77. Nicks identified as partially denatured regions are designated by Greek letters: α, β, γ and δ. pBR322 plasmid DNA was used as a size standard. (B) Distribution of nicks in population of φkF77 molecules. 80 partially denatured molecules were measured. Percentage of molecules with nicks α, β, γ or δ is presented by vertical rectangles with error bars shown. Single-strand DNA fragments numbering corresponds to that in Fig. 1.

The genomic sequence of φkF77 comprises 43,152 bp (accession number NC_012418) and is delineated by two direct terminal repeats of 452 bp. It displays strong similarity at the nucleotide level (up to 90% identity) to other "phiKMV-like viruses". The results of the genome analysis of φkF77 are summarized in the Supplementary Table S1. Analysis of the genome sequences of phiKMV, LKD16 and LUZ19 revealed that these bacteriophages also have the same 14 nucleotide sequence (5′-CGACxxxxxCCTAoh pCTCCGG-3′) repeated at specific locations throughout their genomes (Table 1). Sequences of two other "phiKMV-like viruses", PT2 (NC_011107) and PT5 (NC_011105), became available recently; although we were not able to analyze these phages experimentally, four putative canonical nick regions can be predicted in their genomes. ClustalW-analysis of all nick regions identified gapped motif, with a conserved stretch (CGAC) at position −13 to −10, expanding the initially observed core sequence to 5′-CGACxxxxxCCTAoh pCTCCGG-3′ (Table 1). From this alignment, a clear conservation among all α, β, γ and δ nicks within the entire "phiKMV-like viruses" genus is observed. The subtle differences within the sequences surrounding the conserved nicks might also be a factor contributing to the observed differences in nick frequencies. The genome of phiKMV differs from other phages as it lacks fragments V and VI and the sequence corresponding to nick δ is absent (Fig. 1 and Table 1). This observation indicates that the

Table 1
Identification of the consensus sequences associated with nicks in genomes of the "phiKMV-like viruses".

Nick	Phage	Genome position	Sequence
α	phiKMV	24,130–24,146	CGACCCTGCCCTA*CTCCGGCCTTAAACT
	LUZ19	24,767–24,783	CGACCCTGCCCTA*CTCCGGCCTTAAACC
	LKD16	24,723–24,739	CGACCCTGCCCTA*CTCCGGCCTTAAACC
	φKF77	24,652–24,668	CGACCCTGCCCTA*CTCCGGCCTTAAACC
	PT2	24,493–24,509	CGACCCTGCCCTA*CTCCGGCCTTAAACT
	PT5	24,495–24,511	CGACCCTGCCCTA*CTCCGGCCTTAAACT
β	phiKMV	13,988–14,004	CGACCCTTCCCTA*CTCCGGCCTTAAATC
	LUZ19	14,415–14,431	CGACCCTTCCCTA*CTCCGGCCTTAAATC
	LKD16	14,568–14,584	CGACCCTTCCCTA*CTCCGGCCTTAAATC
	φKF77	14,320–14,336	TGACCCTTCCCTA*CTCCGGCCTTAAATC
	PT2	14,342–14,358	CGACCCTTCCCTA*CTCCGGCCTTAAATC
	PT5	14,353–14,369	CGACCCTTCCCTA*CTCCGGCCTTAAATC
γ	phiKMV	6316–6332	CGACTGGGGCCTA*CTCCGGGGTCAAATC
	LUZ19	6755–6771	CGACCAGGACCTA*CTCCGGGGTCAAATC
	LKD16	6658–6674	CGACCAGGGCCTA*CTCCGGGGTCAAATC
	φKF77	6379–6395	CGACCGGGGCCTA*CTCCGGGGTCAAATC
	PT2	6676–6692	CGACTGGGGCCTA*CTCCGGGGTCAAATC
	PT5	6681–6697	CGACTGGGGCCTA*CTCCGGGGTCAAATC
δ	phiKMV	Deletion	----------
	LUZ19	3132–3148	CGACCCTCGCCTA*CTCCGGGGCCATCGC
	LKD16	3259–3275	CGACCATCGCCTA*CTCCGGGGCCATCGC
	φKF77	2804–2820	CGACCCTCGCCTA*CTCCGGGGCCATCGC
	PT2	3112–3127	CGACCATCGCCTA*CTCCGGGGCCATCGC
	PT5	3112–3127	CGACCCTCGCCTA*CTCCGGGGCCATCGC
		Consensus	CGAC-----CCTA*CTCCGG

Positions of the nicks are indicated with an asterisk.

identified nucleotide sequence is essential for the site-specific nicking of the phage DNA, and shows that "phiKMV-like viruses" can lose at least one of the nicks (δ) without noticeable loss of infectivity.

The identified consensus sequence in "phiKMV-like viruses" differs from that of T5 for which the single-strand interruption consensus sequence is partially conserved for all nicks. Nichols and Donelson (1977a,b) determined the consensus to be Pu_{OH} pGCGC, whereas recent analysis of a complete genome sequence suggests it is 5′-CCC (T)GCGC-3′ (Wang et al., 2005).

Analysis of the genomic localization of the nicks suggests that they are all located in the intergenic regions, which led to the assumption of a putative phage-specific promoter (Lavigne et al., 2003; Ceyssens et al., 2006). It is interesting to note that LKA1, a more distant relative within the "phiKMV-like viruses" lacking DNA homology, does not contain the conserved nicks' sequence and does not produce noticeable ssDNA fragments on agarose gels. However, this phage carries a different, conserved eleven nucleotide sequence (5′-CSGCTGCACTC-3′) in front of genes 5, 7, 18 and 30 (Ceyssens et al., 2006). In the phiKMV-like *Ralstonia solanacearum* phage φRSB1, clear promoter activity associated with conserved intergenic regions was shown. Its consensus sequence also differs from that of the φkF77 nick sites (Kawasaki et al., 2009) but are situated at corresponding genomic locations (i.e. in front of the gene encoding a major capsid protein).

Apart from T5, the "phiKMV-like viruses" are the only group of phages which are reported to have localized nicks in their DNA. Khan et al. (1995) demonstrated that localized single-strand breaks can also be detected in a small part of mature T7 DNA molecules. Unlike T5, "phiKMV-like viruses" do not seem to have minor nicks and the conserved sequence surrounding the nicks is markedly different from that of T5. Although no genes (proteins) responsible for the site-specific interruption of T5 DNA were identified, the involvement of the phage encoded system was clearly demonstrated by the isolation of viable interruption-deficient (lack all nicks) T5 mutants. These mutants appear to define two genes (*sciA* and *sciB*) and they were not deficient for any of the four site-specific endonucleases attributed to T5 (Rogers et al., 1979). It is reasonable to suggest that site-specific nicking of "phiKMV-like viruses" is also phage encoded and is possibly dependent on more than one protein. However, no proteins (e.g. endonucleases, DNA polymerase, terminases) can be directly linked to this occurrence as all these genes found in phiKMV, φkF77, LKD16 and LUZ19 are also present in more distantly related phages (LKA1 and RSB1) that do not display site-specific nicking of their genomes.

Despite the information available to date, the biological significance of the site-specific nicking of the T5 phage DNA remains unknown. Here, we demonstrate that the presence of canonical nicks is a more common/conserved feature of phage genomes than previously thought, which may imply a more fundamental importance of this phenomenon and warrants further investigation.

Materials and methods

Pseudomonas aeruginosa strain PAO1 was used for the propagation φkF77, phiKMV, LKD16 and LUZ19 and cultivations were conducted in rich 2YT medium (Miller, 1972). Bacteriophage DNA was isolated from CsCl gradient purified viral particles using standard protocols (Sambrook et al., 1989). For agarose gel electrophoresis, phage DNA was denatured by alkali (treatment with 0.2 M NaOH for 5 min at room temperature).

ssDNA fragments of φkF77 were isolated from low melting agarose gels as described by Sambrook et al. (1989). Hybridization of these fragments and S1 nuclease analysis were conducted using standard protocols (Sambrook et al., 1989).

For EM analysis of the DNA molecules, a Jeol-100 microscope was available. Sample preparation started using bacteriophage purified by CsCl gradient centrifugation (10^9 pfu/ml), resuspended in a buffer (50 mM Tris–HCl, pH 8.5, 10 mM EDTA) containing formamide. The formamide concentration, temperature and time of denaturing were varied to achieve conditions at which single-strand interruptions in DNA were visualised and at the same time no melting of AT rich regions occurred. For φkF77, DNA denaturation was conducted for 10 min at 37 °C in 85% formamide. Samples were further prepared as described by Davis et al. (1971).

The genome sequencing of phage φkF77 was performed entirely using primer walking, based on available primers and methods used in previous sequencing projects on phiKMV, LKD16 (Lavigne et al., 2003; Ceyssens et al., 2006) and LUZ19. Gene predictions were made by comparative analysis (BLASTP and tBLASTX) to these closely related genomes; regulatory elements were identified as described previously (Ceyssens et al., 2006). Conserved domains were searched using the CDD database (Marchler-Bauer et al., 2007). Conserved pattern detection within phage genome sequences was performed using PHIRE 1.0 (Lavigne et al., 2004). DNA melting temperatures in nicks' regions (basic T_m) were estimated by using Promega biomath calculator found at http://www.promega.com/biomath/calc11.htm.

For preparation of intracellular φkF77 DNA cells were grown and infected as in one-step growth experiment. They were performed according to the method described by Adams (1959) at 30 °C and with PAO1 cells grown to $OD_{600} = 0.3$ ($2\ 10^8$ cell/ml) and infected at MOI of 10. Phage adsorption was allowed at room temperature for 5 min. After adsorption, infected cells were placed into a 30 °C water bath and 1 ml samples were pipetted at various time points on to frozen 0.9% NaCl to stop further DNA synthesis. Samples were further treated and DNA isolated as described by Everett and Lunt (1980). The presence of nick α in the DNA was detected using primer extension analysis with 5′-GGAGGAACAGGGCGATCTG-3′ (LK3 primer; position 25,215–25,533 nt). Primer extension was done with Dream Taq DNA Polymerase (Fermentas) using the following conditions: 94 °C for 2 min and then 30 cycles of 94 °C for 30 s ; 55 °C for 30 s and 70 °C for 90 s The primer extension products were analyzed using QIAxcel System (QIAGEN). A CM-F-RNA program method was used as

recommended by QIAGEN for the analysis of ssDNA. For the control detection of intracellular φkF77 DNA we employed PCR using LK3 (above) and LK1 (5′-GAACTACGCTGGCAAGAACG-3′; 24731–24750 nt) primers.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2009.06.024.

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