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Circular Permutation of the DNA Genome of Temperate Bacteriophage ϕFC1 from *Enterococcus faecalis* KBL703

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Abstract The physical map of bacteriophage ϕFC1 DNA was constructed with the restriction endonucleases *Sal*I, *Bam*HI, *Eco*RI, *Xba*I, and *Ava*I. The 40.5-kb DNA restriction map is shown to be circularly permuted representing the headful packaging mechanism of the phage. The DNA restriction fragments containing the packaging initiation site (*pac*) was localized on the restriction map and the nucleotide sequences of the region were analyzed. Four open reading frames (ORFs), following one another with the same orientation, were found at the region. The 2nd ORF (ORF-ts) has significant amino acid sequence homologies to the previously known terminase small subunits of other bacteriophages. The putative terminase small subunit gene has a presumptive NTP-hydrolysis motif and a helix-turn-helix motif. The cleavage site for the first round of packaging was found to be located at the coding sequence of the putative terminase small subunit gene. The fourth ORF, even if partially sequenced, has a good amino acid sequence homology to the portal vertex proteins of other bacteriophages representing the evolutionarily conserved arrangements of genes near the *pac* site of this bacteriophage, ϕFC1.

Key words: Bacteriophage ϕFC1, restriction map, *pac* site, terminase small subunit

Enterococci have been recognized as one of the normal components of the human intestinal microflora [13]. They are relatively avirulent in healthy persons but they become opportunistically pathogenic in hospitalized or immunocompromised patients [27]. Recently, the pathogenicity of enterococci received an overwhelming attention because of high prevalence of the species in nosocomially infected patients [16, 17] in addition to their rapid development of multiple-drug-resistant strains [12, 13]. The molecular biological research conducted regarding this genus has been mainly concentrated on the sex pheromone and the conjugation mechanism [8]. However, other basic molecular aspects of this genus have not been successfully studied so well, even if they seem to be helpful in understanding and treating disease caused by this microbe.

Bacteriophage ϕFC1 was isolated from the UV-induced lysate of *Enterococcus faecalis* KBL703 [9, 21] and its molecular nature has been characterized in our laboratory [10, 11]. ϕFC1, which belongs to the Siphoviridae, has an icosahedral head, a noncontractile tail, and a double stranded DNA genome with the length of 40.5 kb. In this study, the circular restriction map of 40.5-kb DNA genome was constructed by several type II restriction endonucleases. The packaging initiation site (*pac*) was localized on the restriction map and sequenced. The ORFs located near the *pac* site of ϕFC1 was searched and their amino acid sequences were compared to other bacteriophages.

Materials and Methods

Bacteria and Plasmids

Bacterial strains and plasmids used in this work are listed in Table 1. *Enterococcus faecalis* KBL 703, the lysogenic strain of ϕFC1 was incubated at 37°C in Todd Hewitt broth (THB; Difco, U.S.A.) without shaking.

Induction of Prophage

Bacteriophage ϕFC1 was induced from the culture of *Enterococcus faecalis* KBL 703 cells with UV treatment as described previously [11]. An active 16 h preculture was inoculated as 1% into 100 ml of THB broth and incubated at 37°C for 4 h (turbidity of 0.1 to 0.4), centrifuged at 5,000 ×g for 10 min, resuspended in 50 ml of sterile 0.1 M $MgSO_4$, transferred to a round and flat vessel (30 mm by 120 mm), and irradiated for 15 sec with constant stirring. The UV source was a 15 W germicidal lamp emitting 16

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Table 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics	Reference or source
Enterococcus faecalis KBL 703	Lysogenic for bacteriophage ϕFC1	[9], [11], [21]
E. coli JM109	*recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1* (*lac-proAB*), *F traD36, proAB, lacIq*ZM15	[29]
Plasmids		
pUC19	Amp^r, replicative *ori* of ColE1, *LacZ*	[15]
pFB2	pUC19 carrying 11.6-kb *Bam*HI ϕFC1 fragment	this work
pFB3	pUC19 carrying 7.1-kb *Bam*HI ϕFC1 fragment	this work
pFX1	pUC19 carrying 11-kb *Xba*I ϕFC1 fragment	this work
pFX2	pUC19 carrying 9.7-kb *Xba*I ϕFC1 fragment	this work
pFX3	pUC19 carrying 8.4-kb *Xba*I ϕFC1 fragment	this work
pFX7	pUC19 carrying 1.8-kb *Xba*I ϕFC1 fragment	this work
pFE1	pUC19 carrying 7.7-kb *Eco*RI ϕFC1 fragment	this work
pFE2	pUC19 carrying 7.2-kb *Eco*RI ϕFC1 fragment	this work
pFE3	pUC19 carrying 4.7-kb *Eco*RI ϕFC1 fragment	this work
pFE4	pUC19 carrying 4.2-kb *Eco*RI ϕFC1 fragment	this work
pFE5	pUC19 carrying 4.0-kb *Eco*RI ϕFC1 fragment	this work

ergs/mm^2 at a 40 cm distance. Irradiated cells in 50 ml of 0.1 M $MgSO_4$ were transferred to a new flask containing 50 ml of double-strength THB and incubated at 37°C in darkness for 5 h. Turbidity readings were taken at every 20 min to confirm the lysis of the host cell.

Enzymes and Chemicals

Restriction endonucleases and DNA-modifying enzymes were mostly purchased from Promega (Madison, U.S.A.). The DNA sequencing kit was purchased from U.S. Biochemicals (U.S.A.). [α^{35}-S]dATP and nylon membrane was purchased from Amersham International plc. (U.K.). Other chemicals were mostly provided by Sigma Chemical Co. (St. Louis, U.S.A.).

DNA Preparation

Induced ϕFC1 suspension was concentrated by PEG-8000 and purified according to the methods of Yamamoto *et al.* [28]. DNA extraction from purified phage particles was conducted as described for coliphage λ [19]. Recombinant plasmid DNA from *E. coli* was isolated by the general alkaline lysis technique.

Southern Hybridization

DNA restriction fragments separated on agarose gels were transferred to Hybond-N membranes (Amersham, U.K.) by the capillary method. Probes were labeled with digoxigenin-11-dUTP, and hybridization was performed as recommended by the supplier (Boehringer Mannheim GmbH Biochemica, Germany).

DNA Sequence Analysis and Accession Number

In order to construct clones for nucleotide sequence analysis, nested sets of deletion mutants were generated via the exonuclease III strategy outlined by Sambrook *et al.* [19]. The sequence analysis was performed by the ddNTP chain termination method [20]. Analysis of the DNA primary structure and the DNA or RNA secondary structures were carried out with the computer software package DNASIS (Hitachi software engineering co, Japan). The 700-bp nucleotide sequence data containing the ORFts of ϕFC1 has been deposited in EMBL under the accession number AJ132958. Comparisons of the deduced amino acid sequences of each ORFs near the *pac* region with the proteins in worldwide databases were carried out using the BLASTX algorithm [1].

RESULTS

Basic Frame of Restriction Mapping by *Bam*HI and *Ava*I

Bacteriophage ϕFC1 was harvested and purified from the UV-irradiated lysogenic culture of *Enterococcus faecalis* and the genomic DNA was isolated. To construct the restriction map, appropriate restriction enzymes were selected to those which have relatively small numbers of restriction sites in this phage DNA and from which the produced restriction fragments have relatively big gaps in size that are enough to discriminate easily in the agarose gel electrophoresis. They are *Eco*RI, *Xba*I, *Sal*I, *Bam*HI, and *Ava*I, each of which makes 13, 9, 2, 4, and 3 restriction fragments for the phage DNA (Table 2). Among these enzymes, the basic frame of the map was constructed by *Bam*HI and *Ava*I, which respectively make 4 and 3 fragments each and 7 fragments together (Fig. 1A). The sizes of each fragment were calculated by the comparison with size markers (Fig. 1B). The basic frame is presented in Fig. 1C.

Table 2. Lengths size of fragments produced by single restriction endonuclease digests of ϕFC1 DNA[a].

Fragment	Fragment size (kb)				
	*Bam*HI	*Eco*RI	*Ava*I	*Xba*I	*Sal*I
a	~18.8	7.7	~20	11	~27
b	11.6	7.2	15	9.7	13
c	7.1	4.7	5.5	8.4	2[b]
d	3.0	4.2		3.3	
e		4.0		3.1	
f		3.8[b]		3.1[b]	
g		3.1		2.3	
h		2.5		1.8	
I		2.4		0.6	
j		1.6		0.5	
k		1.1			
l		0.7			
m		0.6			
n		0.5			
Total	40.5	40.3	40.5	40.7	40

[a]Fragment sizes were determined by comparison with appropriate standards in 0.6 to 1.2% agarose gels.
[b]These fragments are submolar (see text) to other bands and hence are not included in the calculation of the total molecular size.

Construction of Restriction Map of ϕFC1 DNA

Most of the DNA fragments produced by a single restriction enzyme from the ϕFC1 (0.9-12 kb) were subcloned at the cloning vector, pUC19, to make the detailed restriction analysis possible. The restriction enzyme digestions (double or triple) and the Southern blotting experiments were carried out a dozen times for the phage DNA or the subcloned DNA during the construction and confirmation of the restriction map. It is important to mention that the innumerable calculation data during the experiments are omitted in this paper. The map was completed as shown in Fig. 2.

Confirmation of the Circular Map by Southern Blotting

The double digested phage DNA by *Bam*HI and *Ava*I was electrophoresed on a 0.7% agarose gel and transferred onto a nylon membrane. The plasmid DNA subclones that overlap each restriction site of *Bam*HI or *Ava*I were labeled and used as the hybridization probes (Fig. 3). Each lane shows that the individual probe was hybridized to two or three fragments that flank the probe fragments. The figure, as a whole, confirms that the restriction map of the phage DNA has a circular shape. In all the double stranded DNA bacteriophages, which have been characterized so far, linear DNAs are in their heads [4]. Therefore, the circular map of bacteriophage DNA has been recognized as the fact that the phage has cyclically permuted DNA molecule resulted from the *pac*-type headful packaging mechanism in the phage head [3, 4, 24]. In this work, the phage ϕFC1 had a circular map and was thought to represent the *pac*-type headful packaging mechanism by the same principle.

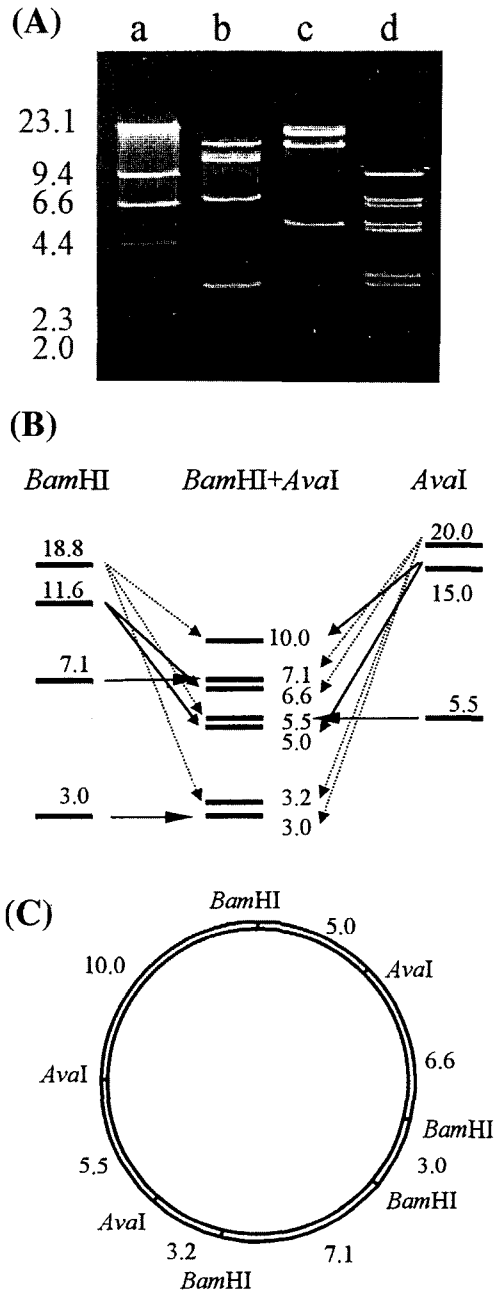

Fig. 1. Construction of the basic frame of phage ϕFC1 DNA restriction map by *Bam*HI and *Ava*I.
(A) Photograph of agarose gel electrophoresis of restriction enzyme digest of ϕFC1 DNA. Lane a, size marker λDNA-*Hin*dIII digest; b, *Bam*HI digest; c, *Ava*I digest; d, *Bam*HI+*Ava*I double digest. The numbers on the left refer to the molecular sizes (in kilobase pairs) of the *Hin*dIII digest of coliphage λ DNA. (B) Schematic diagram of *Bam*HI and *Ava*I digestion. (C) Constructed basic frame. (The order of two *Bam*HI fragments [3.0 and 7.1 kb] is determined by following experiments.)

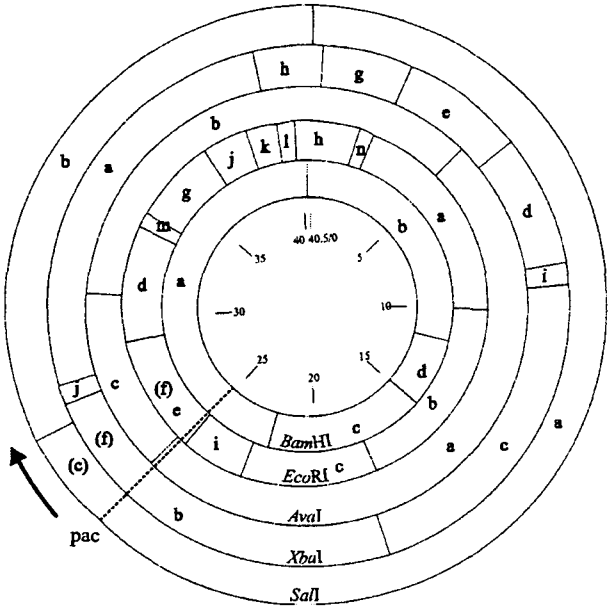

Fig. 2. Restriction map of the ϕFC1 genome.
The sizes and derivation of the fragments are listed in Table 2. The approximate location of *pac*, the site of the first round of packaging of DNA into phage heads is marked. Fragments in parentheses are generated by site-specific cleavage at *pac*. The numbers refer to the size (in kilobase pairs) of the genome. The direction of the packaging is clockwise and represented by an arrow.

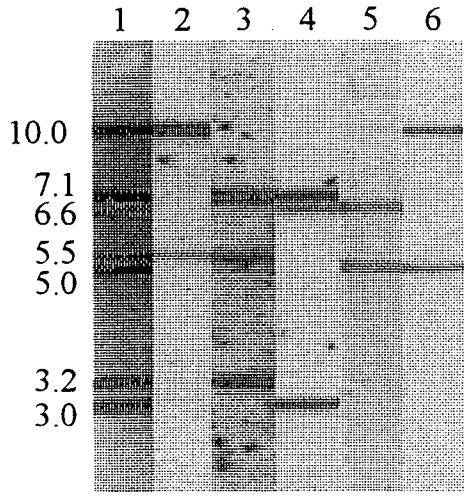

Fig. 3. Southern hybridization analysis of ϕFC1 DNA.
ϕFC1 DNA was digested with *Ava*I and *Bam*HI, electrophoresed on agarose gels, and transferred to nitrocellulose membranes. The DNA bound to the membranes was hybridized with the following Dig-labeled probes: ϕFC1 genomic DNA (lane1), pFX1 (*Xba*I-a) (lane 2), pFX2 (*Xba*I-b) (lane3), pFX3 (*Xba*I-c) (lane 4), pFE1 (*Eco*RI-a) (lane 5), pFX7 (*Xba*I-h) (lane 6). The numbers on the left side refer to fragments sizes (in kilobase pairs).

Presence and Localization of the Packaging Initiation Site

Following the digestion of genomic ϕFC1 DNA with certain restriction enzymes, both submolar bands and

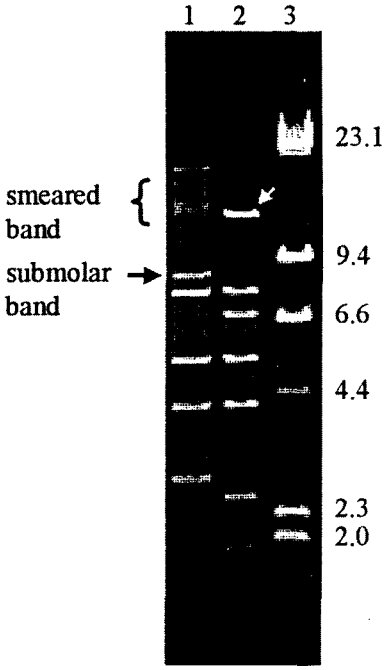

Fig. 4. The presence of submolar bands and smeared bands.
ϕFC1 DNA was digested with restriction enzyme(s) and electrophoresed on 0.7% agarose gels. Lane 1: *Kpn*I, lane 2: *Kpn*I and *Sal*I. The submolar band and the smeared band are indicated. The new large band produced instead of the smeared band are marked by white arrow. The numbers on the right side refers to fragment sizes of size markers, coliphage λDNA-*Hin*dIII digest (lane 3).

smeared bands were definitely observed in the gel electrophoresis of each digest. In Fig. 4, ϕFC1 DNA was digested with *Kpn*I and shows the special bands in the electrophoresis (lane 1). The submolar fragment was explained to be the product of cleavage at the *pac* site of one end of the fragment by the terminase and restriction at the recognition site of the restriction endonuclease at the other end. The smeared fragment turned out to be the set of DNA fragments of similar sizes which was made at the 2nd or later cleavage reaction of the headful packaging mechanism. By calculation for the sizes of the submolar DNA bands at the agarose gel electrophoresis and Southern blotting, the presumptive *pac* site was found to be on the 4.0-kb *Eco*RI fragment. Its location was about 200 bp apart from the *Eco*RI site in which the 4.0-kb and the 2.5-kb *Eco*RI fragments are separated. The calculation data are omitted in this paper because of its complexity.

Sequence Analysis of the *pac*-Containing Region

The nucleotide sequence of the 2.8-kb region containing the *pac* site was determined. The sequencing strategy is shown in Fig. 5. Four ORFs were found at the region even if the first and the fourth were only partially sequenced. The four ORFs were present in a row all with the same orientation. The 2nd and 3rd ORFs encode putative proteins of 20-kDa and 53-kDa molecular weights each. The *pac*

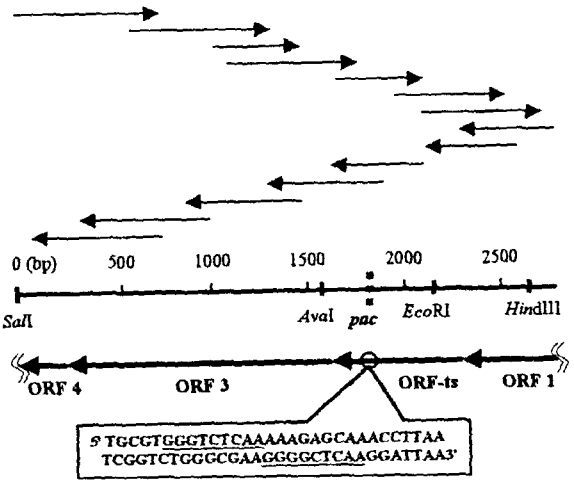

Fig. 5. Sequencing strategy of the region containing *pac* region and the presence of four ORFs.
Each thin arrow at the upper part of the figure represents the direction and content of the various sequence determinations. The sequenced region was scaled at every 500 bps. Four ORFs are represented with thick arrows at the central part. The sequence of presumptive *pac* site located on the ORF-ts is written in the enlarged area as underlined letters. The wavy lines indicate that the ORF1 and ORF4 are not fully sequenced.

site was discovered to be located on the coding sequence of the 2nd ORF.

Amino Acid Sequence Analysis of ORFs Near the *pac* Site

The deduced amino acid sequences of each ORF near the ϕFC1 *pac* region was compared with proteins in available database of amino acid sequence using the BLASTX algorithm. The ORF1 did not show a trace of amino acid sequence homologies with already known proteins. But the 2nd ORF which has a 181 amino acid sequence shows about 40% identity from its Lys (30) to Ile (160) with terminase small subunits from the *B. subtilis* bacteriophage ρ15 or SPP1 or the *Lactobacillus delbrueckii* subsp. *lactis* bacteriophage LL-H. (Fig. 6). Therefore, this ORF (ORF-ts) of ϕFC1 is thought to have the same function of terminase small subunits of other bacteriophages. The ORF3 has 22% amino acid sequence identity with the ORF26 of *Streptococcus thermophilus* temperate bacteriophage O1205 that encodes a theoretical 47-kDa protein (data not shown). The ORF26 is located just downward to the terminase small subunit gene on the phage O1205 genome [22], where the same case of ORF3 was on the ϕFC1 genome. It was apparent that the gene for the terminase large subunit succeeds right after the gene for the terminase small subunit in many other bacteriophages [14] without significant amino acid sequence homologies. The 4th ORF, even if partially sequenced, shows about 40% amino acid sequence homology with a portal vertex protein of the *Streptococcus thermophilus* bacteriophage O125 at the 58 amino acids N-terminal sequenced region.

DISCUSSION

Circular Nature of Phage ϕFC1 DNA Restriction Map

A double stranded DNA bacteriophage has either of two types of maps according to its packaging mechanism [2, 4]. Bacteriophage that has *cos*-type termini similar to the coliphage λ has the same DNA molecules in all the phage particles and holds a linear map. On the other hand, bacteriophage that has *pac*-type termini has circularly permuted DNA in each of the particles and holds a circular map. The phage ϕFC1 DNA represents a complete circular

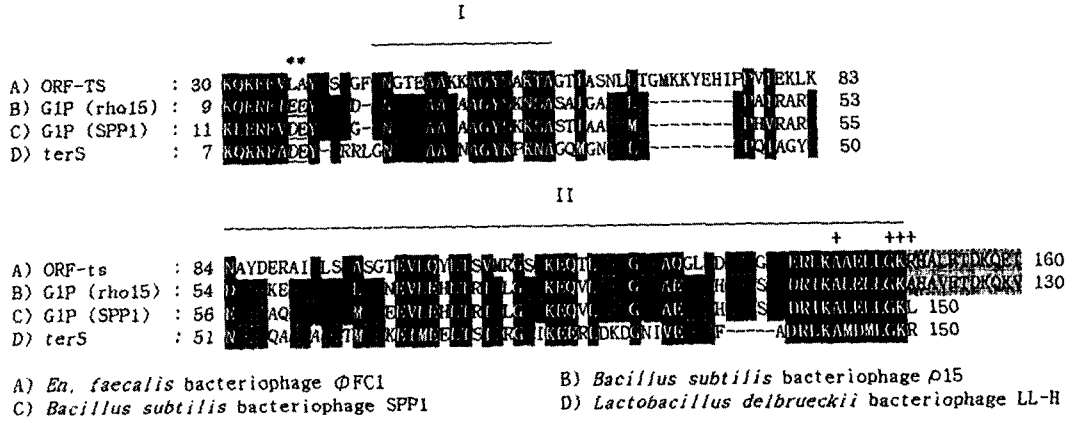

Fig. 6. Alignment of amino acid sequences of ORF2 and other terminase small subunits.
The amino acids are designated by the standard one-letter symbols. The starting and ending positions of each alignment were shown. Comparison of the deduced amino acid sequence of ORF-ts with another proteins were carried out using the BLASTX algorithm. Highly conserved amino acids are shown in the dark background, less well conserved amino acids are in the brighter background. The putative helix-turn-helix DNA binding domain is shown in N-terminal region (segment I), whereas the putative ATP hydrolysis domain is located in the central part (segment II). Asterisks represents the nucleotide binding pocket which was conserved in many bacteriophages but not in this ϕFC1. The putative ATP-binding motif was marked by "+". Dashed lines are empty spaces for the alignment of the amino acids.

restriction map (Fig. 1) representing the *pac*-type packaging mechanism.

Headful Cleavage Mechanism Makes the Submolar or Smeared Fragments

As anticipated from the circular restriction map of this phage DNA, the presence of these submolar bands and the smeared bands altogether could be explained by the fact that ϕFC1 has the processive headful packaging mechanism, originally proposed for coliphage T4 by Streisinger *et al.* [23] and refined for P22 by Tye *et al.* [25]. In the *pac* site containing DNA bacteriophages, the concatemeric DNA produced by the rolling circle replication has about two to five genomic amounts. The *pac* site is used only once at the first round in consecutive cleavages against a concatemeric DNA. The DNA fragment which has one end made by this first cleavage and the other end made by a restriction enzyme presents the unique submolar bands at a gel electrophoresis. Therefore, the direction of packaging is from the *pac* site directed towards the restriction enzyme site and it is located clockwise in this ϕFC1 DNA map (Fig. 2). Fragments which could be generated in a case where proceeding of the packaging were to take place in the counterclockwise direction, were not observed. From the 2nd round of cleavage, packaging machinery cuts the DNA roughly for one headful amount, which is a little more than one genomic (about 104% to 112% of one genomic size in other phages). This redundancy, with the inaccuracy of the headful sizing mechanism, makes the smeared bands at gel electrophoresis. The restriction enzyme *Sal*I cuts off the various ends of *pac*-cleaved DNA fragments in the *Kpn*I-digest of ϕFC1 to make a new large discrete band from the smeared band (lane 2 of Fig. 4).

Arrangements of *pac* Site and Near ORFs

It has been known that the arrangements of various genes in the family Siphoviridae are very well conserved [14]. In many bacteriophages, near the *pac* site, the genes for terminase small subunit, terminase large subunit, and the portal vertex protein are sequentially located in a row. They form a large transcriptional unit of the late genes with downward head and tail assembly proteins. The presence and constitution of three ORFs (ORF2, 3, 4) near the *pac* site of ϕFC1 reveals that this phage follows the above mentioned general rules with strong assurance. In some bacteriophages, there are a few numbers of other short ORFs between the terminase large subunits and the portal proteins. The number of the ORFs represents a variable for each phage but the order is conserved well evolutionarily. The *pac* site (recognition and cleavage site for the packaging initiation) is located at the interior region of the ORF2 like many other phages. It has been known that the *pac* recognition and cleavage sites are composed of GC-rich direct repeats in other bacteriophage [5]. In ϕFC1, a GC-rich direct repeat sequence (5-GGGG(/T) CTCAA-3) with a 30 bp gap is found at the presumptive *pac* region.

Amino Acid Sequence Analysis of Phage ϕFC1 ORFs Present Near the *pac* Site

The terminases are ATP-binding proteins that assemble to form multimeric packaging-protein complexes. In addition to cutting DNA at *pac* or *cos* sites, these proteins perform necessary activities throughout DNA translocation *in vivo* and *in vitro* [3]. They are composed of variable numbers of two subunits. The small subunit, of which the amino acid sequence is well conserved in many phages, consists of the site-specific DNA binding activity and ATP-binding activity. ORF2 has significant amino acid sequence homologies with that of other bacteriophages. It has been anticipated that a helix-turn-helix DNA binding motif [6, 18] is present at the residues 24(Met) to 45(Ala) of *B. subtilis* phage SPP1 (Fig. 6, segment I) [7, 24]. ORF2 of ϕFC1 has good homologies to this region at the 44(Ile) to 65(Ala) and is thought to have the same function. A putative ATP-binding motif "AXXXXGKL" [26] was identified between residues 143 to 150 (marked by "+" in Fig. 6.). The nucleotide binding pocket DE (marked by "*" in Fig. 6.) which was conserved in many other phages was absent at the predicted position. Therefore, a proposal could be made to the fact that some other region of the protein may take control of the nucleotide-binding function.

Acknowledgments

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