Determination of the single strand origin of Shigella sonnei plasmid pKYM

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Abstract

The *Shigella sonnei* plasmid pKYM replicates by a rolling-circle mechanism in *Escherichia coli*. A 571 nucleotides *Hin*cII restriction fragment of the pKYM DNA harbors two potential hairpin loops (I and II). We cloned the fragment into a $-ori$ defective M13 vector phage, M13Δ lac183. The chimera phage, MDKY5, showed a larger plaque size, and increased phage yield and rate of progeny replicative form DNA (RF) synthesis. Rifampicin reduced rate of conversion of the single- to double-stranded RF DNA. In addition, we introduced nucleotide deletions within the cloned pKYM DNA, by *Bal*31 nuclease digestion. Each of the deletion mutants thus constructed was lacking in a sequence containing the hairpin loops and formed smaller plaques. The in vivo analyses revealed that a 136 nucleotides sequence containing the two hairpins I and II is the pKYM minus origin for complementary strand synthesis (single strand origin, referred to as SSO) and harbors a recognition site(s) by host *E. coli* RNA polymerase, for primer RNA synthesis. Moreover, we found a 24 nt sequence, upstream of the SSO domain having 83% homology to the recombination site A (RS_A) which functions in plasmid sitespecific recombination and/or transfer.

Keywords: Single-stranded DNA plasmid; Origin mutant; Deletion; Complementary strand; DNA sequence

1. Introduction

A small cryptic plasmid pKYM with a 2083 nucleotide (nt) double-stranded circular DNA was originally isolated from Gram-negative bacterium *Shigella sonnei* [1,2]. pKYM can grow normally in *Escherichia coli* [1] and belongs to a single-stranded (SS) DNA plasmid family that replicates by a rolling-circle mechanism via SS DNA intermediates [3,4].

As in various Gram-positive bacterial SS DNA plasmids such as pUB110 from *Staphylococcus* (*St.*) *aureus* (see for a review [5]), the pKYM replicon has three key elements for its rolling-circle replication [3,4]: (i) an origin for plus strand synthesis ($+ori$), (ii) a replication protein (*rep*, 321 amino acids long) that nicks a unique site within the $+ori$ domain (located upstream of the *rep* gene), attaches to the 5′ nick terminus via a phosphotyrosine linkage (Tyr-237), displaces the plus strand, and renicks and ligates at the newly generated $+ori$ after one round replication, and (iii) an origin(s) for minus strand replication (SSO).

Contrary to the $+ori/rep$ system, pKYM has no SSO sequences homologous to those of any other SS DNA plasmids [6]. More recently, we [7] have identified a possible pKYM SSO region within an approx. 400 nucleotide (nt) sequence located upstream of the $+ori$; the sequence has two potential secondary structure hairpin loops I and II showing remarkable similarity to $-ori$ sequences of *E. coli* filamentous SS DNA phages fd, f1 and M13 [8–10].

In order to elucidate molecular and evolutional properties of SSO structure, we determined an essential 136 nt sequence of pKYM SSO by a deletion mapping method using *Bal*31 nuclease. Moreover, we found a 24 nt sequence located upstream of the pKYM SSO, which shows 83.3% homology with the conserved plasmid recombination site (RS_A). These results are discussed in relation to the role of secondary structures in the SSO region and to the plasmid transfer.

2. Materials and methods

2.1. Bacteria, plasmids and phages

E. coli strains JM109 [11] and XL1-blue [12], plasmid pKYM, and bacteriophage M13 Δ lac183 were from our laboratory stock [7,13].

2.2. Enzymes and biochemicals

Restriction enzymes, phage T4 DNA ligase, *E. coli* DNA polymerase I (Klenow fragment), *Bal*31 nuclease, and reagents used for dideoxy sequencing were purchased from CalBiochem (USA), Takara Shuzo (Kyoto) and Nippon Gene (Toyama). Buffers for the enzymes were as recommended by the manufacturer. [α-^{35}S]dCTP was from NEN. All other materials were prepared as described previously [14,15].

2.3. Construction of mutant

Double-stranded pKYM DNA was digested with restriction endonuclease *Hin*cII. A 571 nt *Hin*cII fragment (see below) was cloned into a unique *Sma*I site of the M13Δ lac183 vector. Hybrid phages thus obtained were referred to as MDKY5 and MDKY3: the former has the two putative hairpins on the viral strand of M13Δ lac183 (correct direction), whereas the latter has the hairpins on the M13Δ lac183 complementary strand (reversed direction). For deletion analysis, two derivatives MDKY55 and MDKY53 were constructed from MDKY5 (in details, see below). Using these two strains, we introduced deletions into the pKYM SSO by *Bal*31 nuclease digestion, as described previously [16].

2.4. Analysis of phage growth

E. coli JM109 cells were grown at 37°C with shaking in 2YT medium [15]. When the A_{660} of the culture had reached 0.15, phage (input multiplicity, 0.1) was added. At intervals, aliquots were removed, treated with chloroform and then free phage was titrated [17].

2.5. Analysis of DNA synthesis

E. coli JM109 cells were grown at 37°C with shaking in 2YT medium. When the A_{660} of the culture had reached 0.3, phage (input multiplicity, 10) was added. At intervals, phage DNA was extracted by alkaline method [18] and analyzed with 1% agarose gel electrophoresis.

For Southern blotting, *E. coli* JM109 cells were grown at 37°C with shaking in 2YT medium. When the A_{660} of the culture had reached 0.46, rifampicin (100 μg/ml) was added. After 10 min incubation, phage was added (input multiplicity, 10) and the infected culture was incubated for 30 min. The intracellular viral DNA was extracted by alkaline method and electrophoresed on 1% agarose gel, followed by transfer to a nylon membrane (Hybond-N; Amersham). ^{32}P-labeled probes were prepared by nick translation, using the pKYM *Hin*cII fragment. All other procedures for Southern blot hybridization were the same as described previously [2,19].

2.6. DNA sequencing

The nucleotide sequence of the SSO region was determined by the chain termination method [20] using universal vector primers. All other procedures were performed as described previously [21].

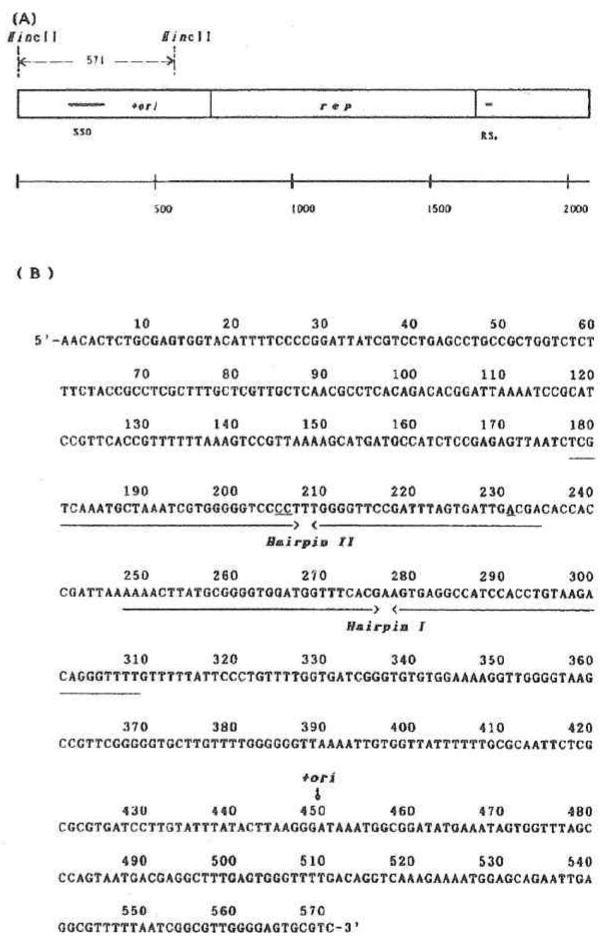

Fig. 1. Genome structure of the pKYM DNA. (A) Gene organization of pKYM is illustrated. SSO; the minus origin for complementary strand synthesis (this study), + *ori*; the plus origin for leading strand replication [3], *rep*; the replication protein [2], RS_A; the plasmid recombination site (see Fig. 3). The *Hin*cII fragment (571 nucleotides) used in this study is highlighted by arrows. (B) DNA sequence of the 571 nucleotides *Hin*cII fragment is presented. The two potential secondary structure hairpin loops I and II are shown by arrows. Three nucleotides revised in this study (see text) are underlined. + *ori*, the plus origin for viral strand synthesis; ↓, the nicking site for *rep* protein.

3. Results

3.1. General properties of pKYM SSO

Fig. 1A shows the pKYM genome structure [2,3]. As described above, pKYM SSO was localized upstream of the $+ori$ domain. In this study, we resequenced pKYM DNA and found three differences in the SSO region with the previously published sequence [2]. The new sequence of 571 nt *Hin*cII fragment containing the SSO is presented in Fig. 1B: an extra dinucleotide CC at nt position 205–206 and an extra A at position 231. Consequently, the complete pKYM DNA is 2086 nt long.

The *Hin*cII fragment contains two possible hairpin loops I and II, which are positioned from nt 248 to 309 and from nt 178 to 234, respectively (Figs. 1B and 2). As indicated by us [7] as well as Seery et al. [22], these secondary structures show extensive sequence homology (74.0%, Fig. 2A), over 140 nt from nt 178 to 317, with those of $-ori$ regions of *E. coli* filamentous phages fd [8], f1 [9], and M13 [10]. In Fig. 2B, the pKYM hairpin loops are compared with those of fd [8].

In addition, like fd, pKYM has a nucleotide T (located at nt 315, see Figs. 1B and 2) at 6 nt upstream of the hairpin I; the fd T has been considered as a starting point for primer RNA synthesis ('primer point') by host *E. coli* RNA polymerase [8].

On the other hand, we found that a 24 nt sequence 5′-AAATAAGaGTATTGGtTTATttTT-3′ located from nt 1698 to 1723 (see Fig. 1A) has 83.3% sequence homology with the conserved 'core' sequence of plasmid recombination site (RS_A) [23]. The RS_A site has been detected in various Gram-positive bacterial plasmids: *St. aureus* plasmids (pE194, pT181, and pUB110) [24], *Streptococcus* (*Sc.*) *agalactiae* plasmid pMV158 [25], and *Lactobacillus* (*Lb.*) plasmids including pLAB1000 from *Lb. hilgardii* [25]), pLB4 from *Lb. plantarum* [26], and pNMO from *Lb.* strain N1f (Kodaira et al., unpublished data). In Fig. 3, the pKYM RS_A sequence is compared with those of Gram-positive bacterial plasmids. Recently, Selinger et al. [27] pointed out that the RS_A domain might be used as an origin for plasmid transfer between bacteria, besides as a recombination site for plasmid cointegrate and/or resolution (see below).

3.2. Cloning of pKYM SSO

To determine pKYM SSO domain, we cloned the 571 nt *Hin*cII fragment (Fig. 1) into a unique *Sma*I site of minus origin defective phage vector, M13Δ lac183. Hybrid phages thus obtained were referred to as MDKY5 and MDKY3 which harbor the two secondary hairpins I and II in correct and reversed directions, respectively, as described in Materials and methods. The MDKY5 strain (equivalent to the M13Δ lac183-4 as in [7]) formed plaques larger than those of the MDKY3 strain, as well as the parental M13Δ lac183 (data not shown), indicating that the *Hin*cII fragment has a SSO activity.

3.3. Growth properties of MDKY5 strain

In order to confirm the SSO activity, we performed phage growth experiments. When *E. coli* JM109 cells were infected with the MDKY5 strain at 37°C, the phage

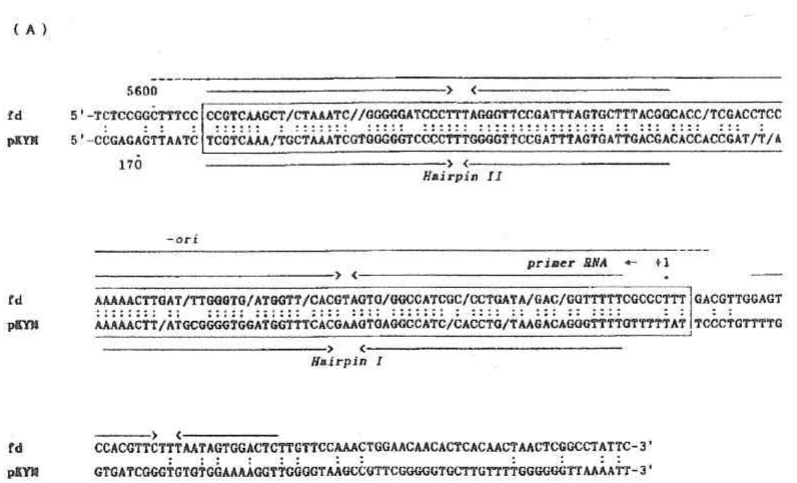

Fig. 2. Structure of the pKYM SSO. (A) DNA sequence of the pKYM SSO (this study) is compared with the $-ori$ of *E. coli* filamentous phage fd [8]. Identical nucleotides between fd and pKYM are indicated by (:). Slashes represent gaps inserted to align nucleotides for maximal homology. The two putative secondary hairpin loops I and II are indicated with arrows. The fd $-ori$ domain is boxed by dotted line; its sequences recognized by RNA polymerase are shown by horizontal line [8]. +1, a putative starting point for fd primer RNA synthesis [8]. (B) Secondary structures of pKYM SSO and fd $-ori$ domains are compared. Sequences of pKYM and fd are from this study and [8], respectively. Nucleotides of fd identical to those of pKYM are presented by capital letters.

(B)

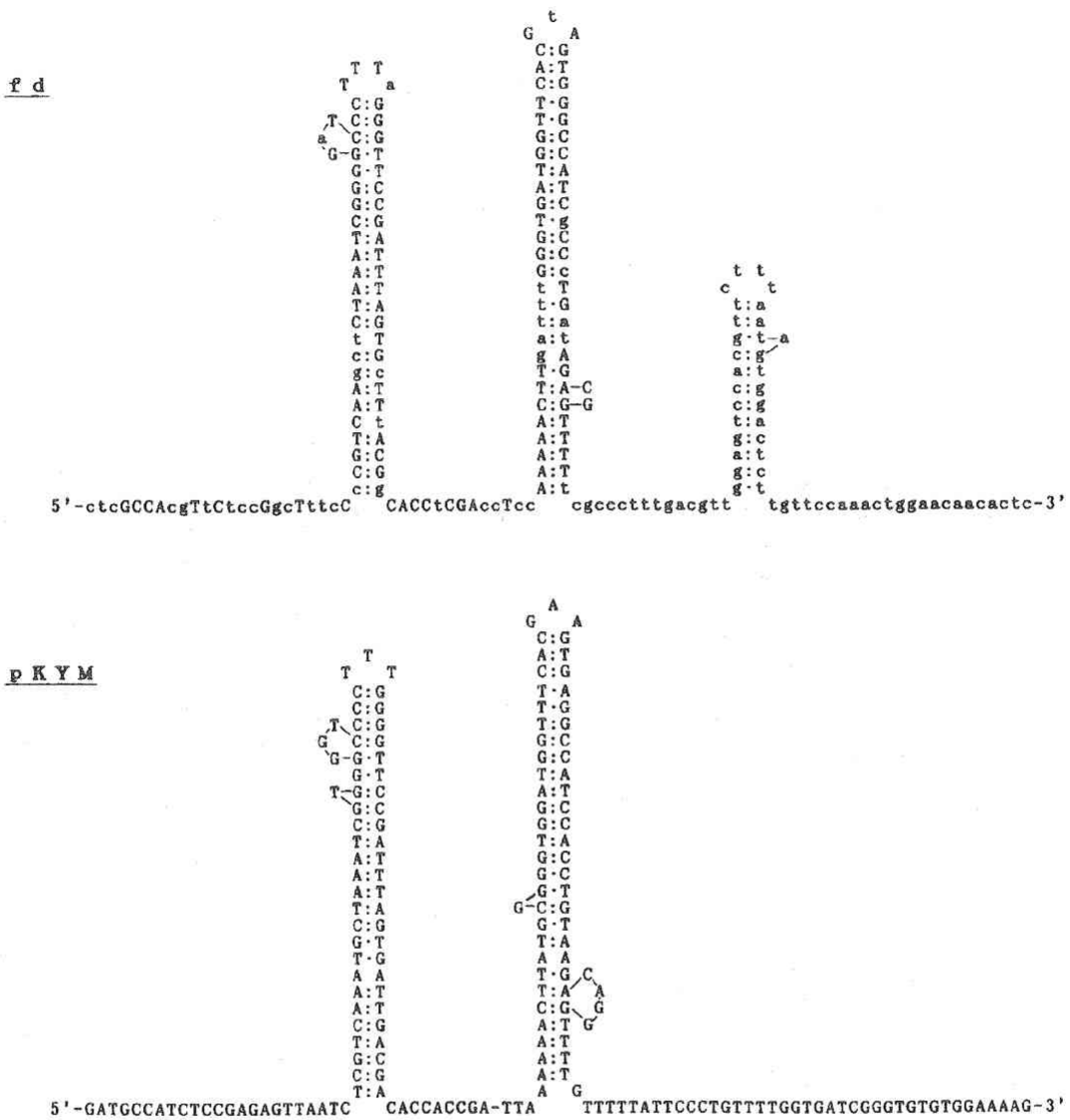

Fig. 2 (continued).

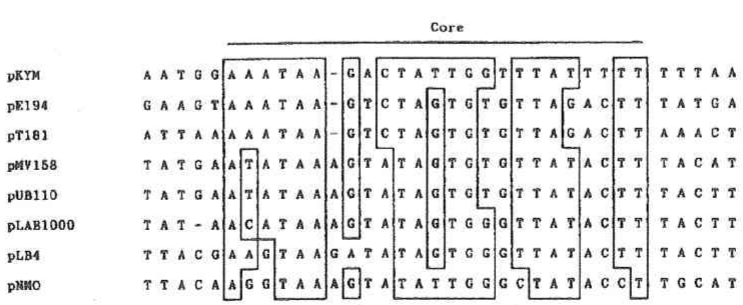

Fig. 3. Similarities among recombination sites of pKYM and other Gram-positive bacterial plasmids. Sequences of pKYM, pE194, pT181, pMV15 and, pUB110, pLAB1000, pLB4, and pNMO are from [2,30,24,31,24,26] and Kodaira et al. (unpublished data), respectively. Core sequence is represented by horizontal line. Identical nucleotides are boxed. Imperfect inverted repeats are indicated by arrows.

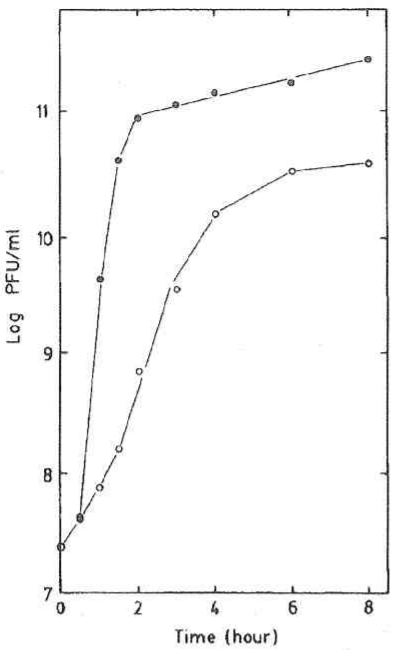

Fig. 4. Analysis of phage growth. Cells of *E. coli* JM109 ($3.0 \cdot 10^8$/ml) grown at 37°C were infected with phage (input multiplicity, 0.1) in 2YT medium [13]. At intervals, aliquots were removed, treated with chloroform and then free phage was titrated. ○, M13Δ *lac*183; ●, MDKY5.

yield was about one order higher at 8 h after infection than that of the parental M13Δ lac183 (Fig. 4) as well as the MDKY3 strain (data not shown).

The SSO region of SS DNA plasmid is utilized in the conversion of newly synthesized closed circular plus strand into the parental replicative form (RF) DNA [3,5]. To study effects of the pKYM SSO on the hybrid viral DNA replication in vivo, rate of the RF synthesis was measured in *E. coli* JM109. The cells were infected with the MDKY5 strain and the intracellular viral DNA was subjected to agarose gel electrophoresis analysis. Fig. 5A shows RF synthesis of the MDKY5 strain comparing with that of the parental M13Δ *lac*183. Amount of MDKY5 RF DNA accumulated after infection was increased in parallel with increase in phage yield (see Fig. 4).

In comparison with fd −*ori* [8], we examined dependency of the pKYM SSO on *E. coli* RNA polymerase. *E. coli* JM109 cells treated with rifampicin (100 μg/ml), an inhibitor of RNA polymerase, were infected with MDKY5 and then the intracelluar viral DNA was analyzed using Southern blot hybridization, as described in Materials and methods. As shown in Fig. 5B (lane o), MDKY5 SS DNA was accumulated after rifampicin treatment and its RF formation was strongly inhibited, demonstrating that SS DNA molecules were intermediates for its rolling-circle replication; linear-like molecules migrated slower than closed-circular DNA (RFI) may be derived from a cryptic origin(s) in M13Δ *lac*183 DNA which is probably independent of RNA polymerase [14,16]. In the presence of chloramphenicol (30 and 150 μg/ml), no such SS DNA intermediates were detected (Fig. 5B, lanes m and n, respectively).

These observations suggest that the 571 nt sequence contains a unique pKYM SSO domain and harbors a recognition site(s) of *E. coli* RNA polymerase which is involved in primer RNA synthesis for complementary strand replication. In the pKYM hairpins, we found putative transcription initiation signals, at −35 and −10 regions (see below).

3.4. pKYM SSO mutants

To elucidate to what extent the secondary structure of the hairpin loops I and II is essential, we introduced

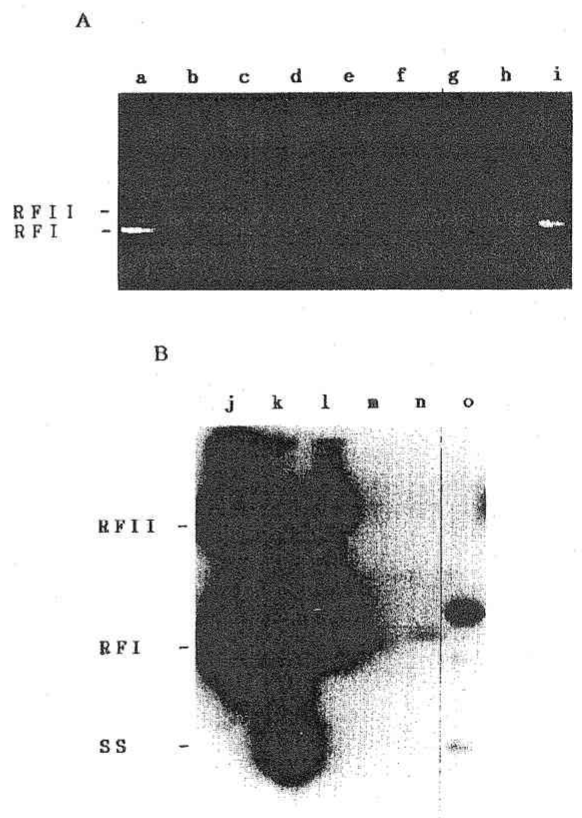

Fig. 5. Rate of synthesis of MDKY5 RF DNA. (A) Cells of *E. coli* JM109 ($5.0 \cdot 10^8$/ml) grown at 37°C were infected with phage (input multiplicity, 10) at 37°C in 2YT medium [13]. At 20, 40, 60, and 90 min postinfection, the intracellular viral DNA was extracted and subjected to 1% agarose gel electrophoresis. a, control MDKY5 RF DNA; b, c, d, and e, M13Δ *lac*183 at 20, 40, 60, and 90 min, respectively; f, g, h, and i, MDKY5 at 20, 40, 60, and 90 min, respectively. (B) Cells of *E. coli* JM109 ($5.0 \cdot 10^8$/ml) grown at 37°C in 2YT medium [13] were treated with rifampicin or chloramphenicol for 10 min and then phage was added (input multiplicity, 10). After 30 min incubation, the intracellular viral DNA was extracted and subjected to 1% agarose gel electrophoresis. The viral DNA was detected with Southern blot hybridization as described in Materials and methods. j, MDKY5 RF DNA; k, MDKY5 SS DNA; l, no inhibitor, m, chloramphenicol (30 μg/ml); n, chloramphenicol (150 μg/ml); o, rifampicin (100 μg/ml).

nucleotide deletions within the pKYM SSO by the method of *Bal*31 nuclease digestion [16].

For deletion mapping, we constructed two derivatives of the MDKY5 strain, as probes (for details, see Fig. 6). One derivative, MDKY53, has 420 nt sequence spanning from *Hin*cII (located at nt 1) to *Acc*II (at nt 421) sites, and the other derivative, MDKY55, contains 284 nt sequence from *Dra*I (at nt 137) to *Acc*II (at nt 421) sites (both of the derivatives have restriction enzyme sites for introduction of deletion, at their flanking regions). The two strains formed plaques as large as those of the parental MDKY5 (data not shown).

Deletion mutants thus obtained were referred to as *del*MDKY53 or *del*MDKY55. To determine the mutation sites precisely, the mutants were subjected to sequence analysis by the chain termination method [20] using universal vector primers. Each mutant had an expected mutation within the secondary structures. In Fig. 7, their mutation sites are summarized and compared with the fd $-ori$ sequence.

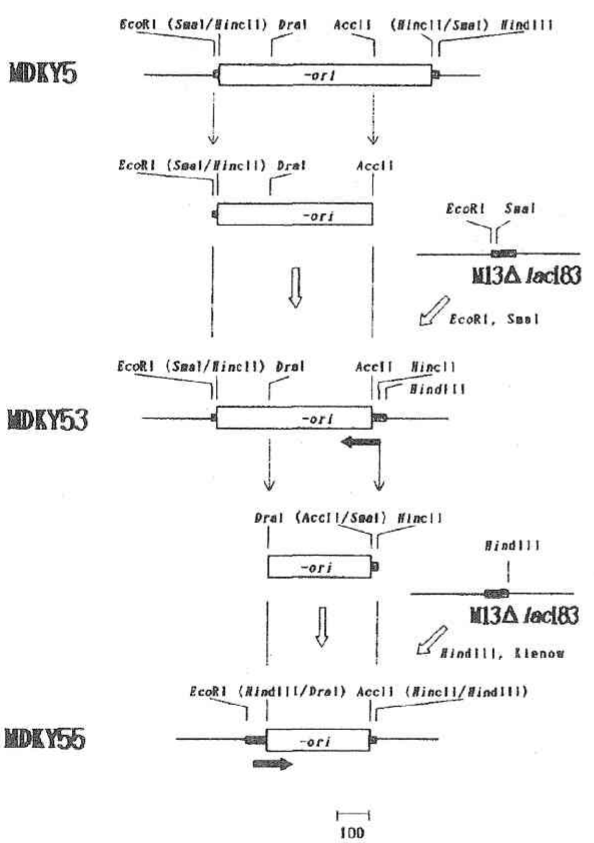

Fig. 6. Construction of MDKY53 and MDKY55 derivatives. MDKY5 RF DNA (see text) was cut by restriction enzymes *Eco*RI and *Acc*II and the *Eco*RI/*Acc*II fragment was recloned into M13Δ *lac*183 using its *Eco*RI/*Sma*I sites. The resulted derivative was referred to as MDKY53. A *Dra*I/*Hin*cII fragment of MDKY53 was recloned into M13Δ *lac*183 using its endfilled *Hin*dIII site and a MDKY55 derivative was obtained. Regions containing multi-cloning site are indicated by dashed box.

Mutants missing a sequence within the two hairpins formed smaller plaques like M13Δ *lac*183: (1) two mutants *del*MDKY55-246 and -207 had 16 and 26 nt deletions, respectively, within the 5′ stem of hairpin II, (2) *del*MDKY55-227 lost the two hairpins entirely, but still had the primer point (see above), whereas (3) *del*MDKY53-129 and -123 mutants missed almost all of the SSO region containing the 3′ stem of hairpin II, a region between the hairpins I and II ('loop space'), the hairpin I, and the primer point, (4) *del*MDKY53-139 was lacking in the 3′ region of loop space and the hairpin I, besides the primer point, (5) *del*MDKY53-125 and -147 mutants missed almost all of the hairpin I and the primer point.

Contrary to these mutants yielding smaller plaques, *del*MDKY53-163 formed plaques as large as those of the parental MDKY53; the mutant lost the primer point and 3 nt (5′-TTT-3′) at the base in the 3′ stem of hairpin I. Other two mutants *del*MDKY53-2 and -9, lacking in the primer point but not the secondary structures, also produced larger plaques. In the case of 5′ mutant, *del*MDYK55-197 formed larger plaques; it still had the entire secondary structures and the 5′ flanking 7 nt 5′-GTTAATC-3′.

These sequencing results revealed that the pKYM SSO consists of 136 nt located from nt 171 to 306 (see Figs. 1 and 7) which comprise the two secondary hairpins I and II.

4. Discussion

An abundance of plasmid species replicating by a rolling-circle mechanism have been found in various Gram-positive and -negative bacteria. Contrary to their $+ori$/Rep systems, structure and function of the SSO domains are remarkably divergent; they are different in DNA sequence, albeit rich in distinct secondary structures. This is mainly due to different dependency of the SSO domains on host replication proteins, including DNA primase, RNA polymerase, and so on. They can be divided into several groups, such as palA, BA3 (or palU), BAA1, fd, ϕX174, G4 and other types [24,25], but their evolutionary relationship is at present unclear.

In this study, the SSO domain of a small cryptic *Shigella sonnei* plasmid, pKYM (2086 nt DNA), was determined within a 136 nt sequence which contains the two secondary structure hairpin loops I and II (Fig. 2B) with 75.4% homology with the $-ori$ of *E. coli* filamentous phage fd (Fig. 2A). The pKYM SSO sufficiently functioned in a $-ori$ defective phage vector M13Δ *lac*183 (Figs. 4 and 5A) and its activity was inhibited by rifampicin, an inhibitor against to *E. coli* RNA polymerase (Fig. 5B).

These in vivo analyses demonstrated that the 136 nt secondary structures (the hairpin I, the loop space, and the hairpin II) are essential for recognition by *E. coli* RNA polymerase, which synthesizes a primer RNA for comple-

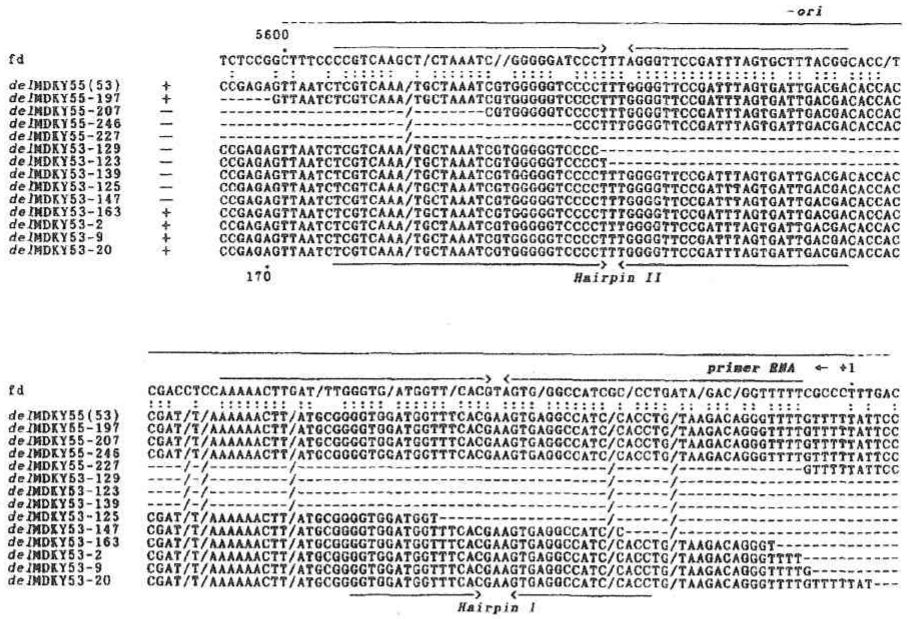

Fig. 7. Nucleotide sequences of the SSO mutants. Identical nucleotides between fd and pKYM are indicated by (:). Slashes represent gaps inserted to align nucleotides for homology. Putative secondary hairpin loops are shown with arrows. Deletion mutants of pKYM are described in the text. Deleted nucleotides are indicated with dashes. +, large plaques; −, small plaques. Other symbols are shown in Fig. 2.

mentary strand replication. It is interesting that the pKYM hairpins have possible transcription initiation signals; a sequence 5′-TTGACG-3′ (positioned from nt 228 to 233, see Fig. 1) for the −35 region is located on the 3′ stem of hairpin II, whereas a sequence 5′-TATGcgG-3′ (from nt 254 to 259) for the −10 region is positioned on the 5′ stem of hairpin I. The two putative signals separated by definite 20 nt are shown in Fig. 8, in comparison with well characterized promoters of *E. coli* phages fd [28] and ϕX174 [29]. Upon recognition by *E. coli* RNA polymerase of these sequences for primer RNA synthesis, nucleotide A located at nt 287 and/or dinucleotide GG at position 283–284 are most probable initiation points. This observation coincides well with the fact that the three deletion mutants *del*MDKY53-163, -9, and -2 are lacking in the nucleotide T at nt 315 (see Fig. 7).

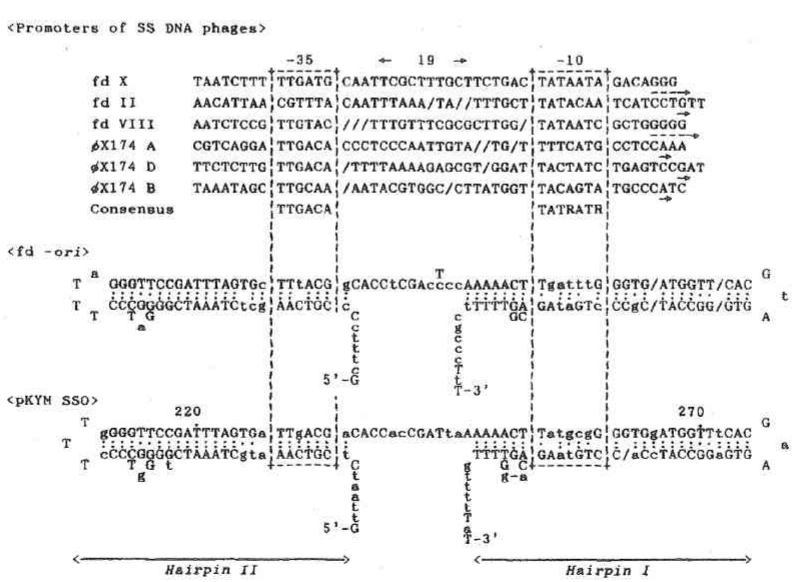

Fig. 8. Transcription initiation signals of pKYM SSO. Promoters of fd and ϕX174 are from [28,29], respectively (mRNA starting points are represented by small arrows and slashes represent gaps inserted to align nucleotides for maximal homology). The sequences of fd −*ori* and pKYM SSO are from [8] and this study, respectively. The two putative secondary hairpins I and II are arrowed.

Previously, Yasukawa et al. [3] indicated that genetic organization of the pKYM $+ori/rep$ system resembles that of the pUB110 family, such as pC194 (from *St. aureus*) and suggested that pKYM as a plasmid of the pUB110 family was transferred accidentally to Gram-negative bacterium.

In pKYM DNA, we found a RS_A-like sequence with 83% homology with the conserved RS_A domain (core sequence) found in various Gram-positive bacterial plasmids (see above). The core sequence plays an important role in site-specific recombination for plasmid cointegration and/or resolution as a unique target site of plasmid-encoding *rec*A-independent recombinase, Pre (plasmid recombination) [24]. In *Staphylococcus* plasmids such as pT181 and pE194, pre-mediated recombination occurs between inter (as well as intra) molecular RS_A sites [11]. Recently, Selinger et al. [27] indicated that nonconjugative plasmid pUB110 (from *St. aureus*) or pBC1 (from *Bacillus cereu*) possessing Pre/RS_A system can be transferred to other strain through its RS_A site, with the help of a conjugative plasmid. Thus, the putative transfer of pKYM to a Gram-negative bacterium [3] might have been mediated by its RS_A sequence and have acquired a SSO domain which can be recognized by Gram-negative bacterial RNA polymerase, as in *E. coli* filamentous phages such as fd, but not in spherical phages G4 and ϕX174 requiring bacterial primase [14,15].

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