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**J Y Jeong, G M Yoon, H S Seo, M J Cho, H Sakai, T Komano and J D Bahk**

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# Functional Features of an *ssi* Signal of Plasmid pGKV21 in *Escherichia coli*

JIN-YONG JEONG,[1] GYEONG-MEE YOON,[1] HAK-SOO SEO,[1] MOO-JE CHO,[1] HIROSHI SAKAI,[2] TOHRU KOMANO,[2] AND JEONG-DONG BAHK[1]*

*Department of Biochemistry, Gyeongsang National University, Chinju 660-701, Korea,[1] and Laboratory of Biochemistry, Department of Agricultural Chemistry, Kyoto University, Kyoto 606-01, Japan[2]*

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**A single-strand initiation (*ssi*) signal was detected on the *Lactococcus lactis* plasmid pGKV21 containing the replicon of pWV01 by its ability to complement the poor growth of an M13 phage derivative (M13Δ*lac*182) lacking the complementary-strand origin in *Escherichia coli*. This *ssi* signal was situated at the 229-nucleotide (nt) *Dde*I-*Dra*I fragment and located within the 109 nt upstream of the nick site of the putative plus origin. SSI activity is orientation specific with respect to the direction of replication. We constructed an *ssi* signal-deleted plasmid and then examined the effects of the *ssi* signal on the conversion of the single-stranded replication intermediate to double-stranded plasmid DNA in *E. coli*. The plasmid lacking an *ssi* signal accumulated much more plasmid single-stranded DNA than the wild-type plasmid did. Moreover, deletion of this region caused a great reduction in plasmid copy number or plasmid maintenance. These results suggest that in *E. coli*, this *ssi* signal directs its lagging-strand synthesis as a minus origin of plasmid pGKV21. Primer RNA synthesis in vitro suggests that *E. coli* RNA polymerase directly recognizes the 229-nt *ssi* signal and synthesizes primer RNA dependent on the presence of *E. coli* single-stranded DNA binding (SSB) protein. This region contains two stem-loop structures, stem-loop I and stem-loop II. Deletion of stem-loop I portion results in loss of priming activity by *E. coli* RNA polymerase, suggesting that stem-loop I portion is essential for priming by *E. coli* RNA polymerase on the SSB-coated single-stranded DNA template.**

Primer RNA synthesis for the replication of *Escherichia coli* single-stranded DNA (ssDNA) phages may be accomplished by one of the three different modes of priming (19). Initiation mechanisms of complementary-strand synthesis have been distinguished on the basis of the minus-origin sequences and host function requirements. In each case, an RNA primer is synthesized prior to the initiation of DNA synthesis. This RNA primer is synthesized by primase and RNA polymerase in case of G4 and M13 phages, respectively (4, 7). In ϕX174, conversion of ssDNA to double-stranded DNA (dsDNA) requires the formation of a primosome, a multiprotein complex composed of at least seven host proteins (29). Primer RNAs are synthesized at multiple sites located throughout the ϕX174 genome due to the mobile nature of this protein complex (1, 26, 28). It was found that many plasmids isolated from *E. coli* contained specific nucleotide sequences required for priming DNA synthesis on an ssDNA template. These sequences have been called the single-strand initiation (*ssi*) signals. These *ssi* signals were identified by their ability to complement the poor growth of an ssDNA phage in which a great part of minus origin had been deleted (17, 27). The *ssi* signals of *E. coli* plasmids were also classified into four major groups with respect to their priming mode: (i) G4 type (3, 23, 24, 32), (ii) ϕX174 type (2, 15, 23–25), (iii) ABC type (22), and (iv) plasmid-specific type (RSF1010) (12–14).

Many reports have described that plasmids isolated from gram-positive bacteria replicate by the rolling-circle-type mechanism, typical of the *E. coli* ssDNA phages. These plasmids, referred to as ssDNA plasmids (8, 10), also contain minus origins which are involved in the conversion of the single-stranded replication intermediate to the double-stranded plasmid DNA form. Usually minus origins have been reported to use host RNA polymerase to initiate lagging-strand synthesis and function only in a limited number of host bacteria (10).

Plasmid pGKV21 is a shuttle vector containing the replicon of lactococcal plasmid pWV01 (18, 33) which allows the isolation of promoter and transcription termination signals in lactic streptococci. Plasmid pWV01 has been shown to be able to replicate in both gram-positive and gram-negative bacteria, including *E. coli* (20, 30). Demonstration of functional roles of replication elements during the pGKV21 replication in gram-negative bacteria, not in gram-positive bacteria, is important to understand the replication mechanism of pGKV21 as a broad-host-range plasmid.

We have previously reported that plasmid pGKV21 contains a 229-nucleotide (nt) *ssi* signal (16). This *ssi* signal was located within the 109 nt upstream of the nick site of the putative plus origin. Also, this *ssi* signal could form two potential stem-loop structures, stem-loops I and II, as is the case for almost all other *ssi* signals. Moreover, the 229-nt *ssi* signal was orientation dependent for SSI activity in the direction *Dde*I to *Dra*I, as shown in Fig. 1.

In this study, we constructed an *ssi* signal-deleted plasmid and then examined the effects of the *ssi* signal on replication activity in *E. coli*. Furthermore, to delineate the essential structural features of the *ssi* signal, we carried out extensive mutational and deletional analyses of the *ssi* signal. The results suggest that *E. coli* RNA polymerase generates RNA primers on the *ssi* signal in the presence of *E. coli* ssDNA binding (SSB) protein, and the stem-loop I portion is essential for priming by *E. coli* RNA polymerase on the SSB-coated ssDNA template.

## MATERIALS AND METHODS

**Bacterial, phage, and plasmid strains.** *E. coli* TG1 was used as the host bacterium for the filamentous phage vector. Plasmid pGKV21 is a derivative of

* Corresponding author. Phone: 82-591-751-5959. Fax: 82-591-759-9363. E-mail: jdbahk@nongae.gsnu.ac.kr.

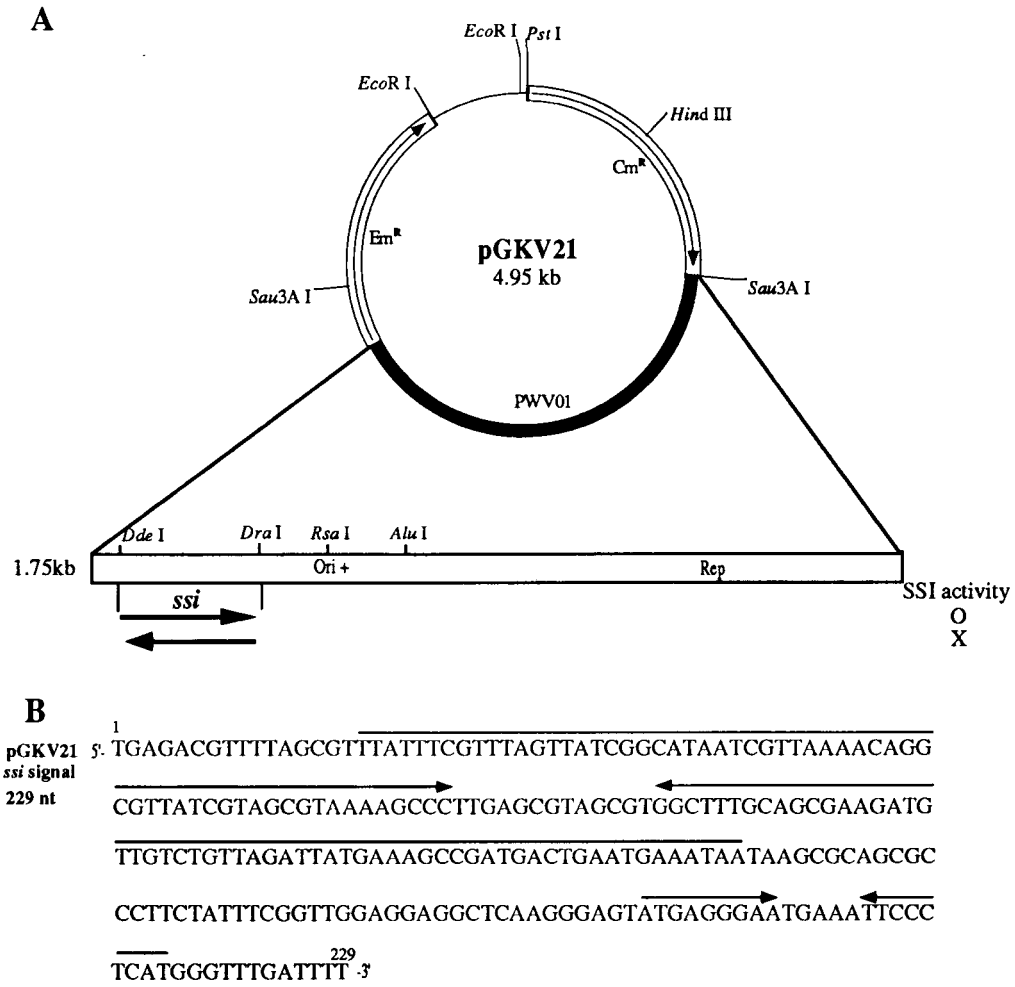


FIG. 1. Location and nucleotide sequence of the *ssi* signal of plasmid pGKV21. (A) Physical map of pGKV21 is based on the data reported by van der Vossen et al. (33). The thick solid line indicates a part of pWV01. Positions of the chloramphenicol resistance ($Cm^R$) and erythromycin resistance ($Em^R$) genes are drawn in open boxes containing arrows. The horizontal open box below indicates the map of pWV01. The Rep (region encoding Rep protein) and Ori+ (plus origin) sites are represented by sequence similarity with the RepB and plus origin of plasmid pLS1, respectively. The thick arrows at the left of the open box represent the orientation of SSI function. O and X indicate the presence and absence, respectively, of the SSI activity of the *ssi* signal. (B) Nucleotide sequence of an *ssi* signal in pGKV21. The signal sequence consists of the *Dde*I-*Dra*I segment which has the potential to form two stem-loop structures. The palindromic sequences are indicated by arrows above the nucleotide sequence.

the lactococcal plasmid pWV01 (18, 33), which is a broad-host-range plasmid able to replicate in both gram-positive and gram-negative bacteria. The filamentous phage vector M13Δ*lac*182, an *ssi* signal probe vector, is a mutant M13 phage vector with a deletion of the minus-strand origin (17, 27).

**Enzymes and reagents.** Restriction endonucleases, BAL 31 nuclease, *E. coli* SSB protein, and other DNA-modifying enzymes were purchased from Promega Corporation (Madison, Wis.). Bovine serum albumin and nucleoside triphosphates (ATP, CTP, GTP, and UTP) were obtained from Sigma (St. Louis, Mo.). [α-$^{32}$P]GTP (400 Ci/mmol) was obtained from Amersham Life Science (Buckinghamshire, England), and *E. coli* RNA polymerase was obtained from Boehringer (Mannheim, Germany).

**Phage growth assay.** The growth assay was done as previously described (2). Bacterial growth after phage infection was carried out as 37°C in LG-B medium (pH 7.2) containing, per liter, 10 g of Bacto Tryptone, 5 g of yeast extract, and 8 g of NaCl, to which were added 5 ml of 20% glucose, 1 ml of 1 M $CaCl_2$, and 10 ml of 0.2% thymine after sterilization. Phage titer was calculated at 0, 2, 4, and 8 h after infection of *E. coli* TG1 cells. Each portion of the phage-infected culture was withdrawn and subjected to serial dilution of $10^{-7}$, $10^{-9}$, and $10^{-11}$. The diluted suspensions of the culture were plated with host strain *E. coli* TG1.

**In vitro primer RNA synthesis.** Primer RNA synthesis was determined as previously described (7, 11). All reactions for in vitro primer RNA synthesis were carried out in 20 μl of reaction mixture containing 0.2 μg of phage ssDNA, 4 μg (0 to 8 μg in Fig. 5A) of *E. coli* SSB protein, 40 mM HEPES-KOH (pH 7.5), 5 mM $MgCl_2$, 130 mM KCl, 0.02 mg of bovine serum albumin per ml, 500 μM ATP, CTP, and UTP, 50 μM GTP, and 20 μCi of [α-$^{32}$P]GTP (400 Ci/mmol). Reactions were started by adding 1 U of *E. coli* RNA polymerase. After incubation for 20 min at 30°C, reactions were stopped by addition of 80 μl of stop solution, which resulted in final concentrations of 80 mM NaCl and 10 mM EDTA. The nucleic acids were precipitated with 250 μl of ethanol, dried, and resuspended in 10 μl of 80% formamide dye containing bromophenol blue and xylene cyanol. After boiling for 2 min, samples were put on ice immediately and electrophoresed on a 12% polyacrylamide–7 M urea gel in 1× Tris-borate-EDTA buffer. After electrophoresis, the gel was soaked in 10% methanol–10% acetic acid mixture for 10 min, dried, and autoradiographed with intensifying screens.

**Construction of the *ssi* signal mutants.** The replicative form (RF) DNA of recombinant phage DNA carrying the 229-nt *ssi* signal of pGKV21 was mutagenized either by BAL 31 nuclease deletion or by modified PCR random substitution. For BAL 31 nuclease deletion, the RF DNA was linearized by *Eco*RI or *Hin*dIII and digested with BAL 31 nuclease at 30°C for different lengths of time. The linearized and digested DNAs were filled in with Klenow fragment and further digested by *Hin*dIII for *Eco*RI-digested DNA or by *Eco*RI for *Hin*dIII-digested DNA. The products were electroeluted from the agarose gel. The eluted DNA was ligated with M13Δ*lac*182 RF DNA digested by *Sma*I and *Hin*dIII for the *Eco*RI/BAL 31-treated DNA or with that digested by *Sma*I and *Eco*RI for the *Hin*dIII/BAL 31-treated DNA. PCR-directed substitution mutagenesis was then performed by the method of Leung et al. (21), with minor modification. The PCR mixtures contained 10 μl of template DNA (5 ng/ml), 10 μl of 10× *Taq* DNA polymerase buffer, 1 μl of 1 M β-mercaptoethanol, 10 μl of dimethyl sulfoxide, 10 μl of 5 mM $MnCl_2$, 1.5 μl each of T3 (forward) and T7

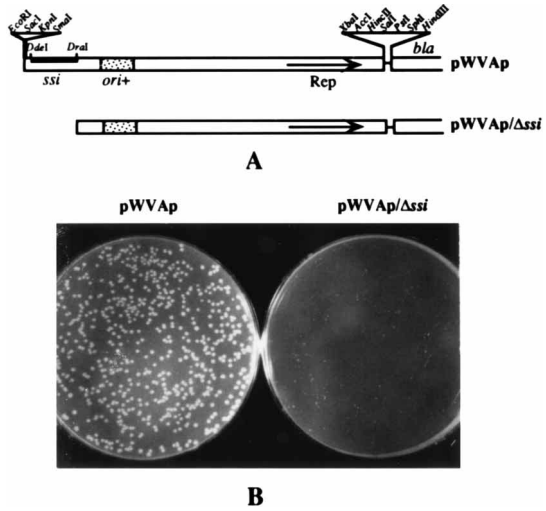


FIG. 2. Construction of an *ssi* signal-deleted plasmid. (A) Physical map of plasmids pWVAp and pWVAp/Δ*ssi*. (B) Colonies of *E. coli* harboring either pWVAp or pWVAp/Δ*ssi.*

(reverse) primers (30 ng/μl), 10 μl of 10 mM each dGTP, dCTP, and dTTP, 10 μl of 2 mM dATP, and 16 μl of $H_2O$. DNA sample was denatured to separate strands at 90°C for 30 s. The denatured DNA single strands were annealed with forward and reverse primers at 50°C for 15 s and chain elongated by *Taq* DNA polymerase at 72°C for 1 min. They were then cycled 30 times. The deletion endpoints and substituted nucleotides were confirmed by dideoxy chain termination sequencing using an ABI 373A automatic DNA sequencer (Applied Biosystems, Foster City, Calif.).

**Plasmid maintenance.** Cells containing plasmids were grown in LB medium in the presence of ampicillin. In the late logarithmic growth phase, cells were diluted to an optical density at 650 nm of 0.005 in nonselective LB medium and incubated at 37°C. Cultures were diluted to $10^{-3}$-fold at 110-min intervals, corresponding to a period of five generations. Approximately $10^4$ cells taken at each dilution step were plated on a nonselective LB agar for estimation of the number of generations that the cells had grown under nonselective conditions. Subsequently, 100 colonies were toothpicked and transferred to ampicillin-containing plates for estimation of the percentage of cells that still contained the plasmid.

## RESULTS

**Functional contribution of the *ssi* signal to the plasmid genome.** The *ssi* signal of plasmid pGKV21 is located on a 229-nt *Dde*I-*Dra*I fragment (Fig. 1). This *ssi* signal was detected by the plaque morphology procedure described previously (16). The plasmid DNA of pGKV21 was cleaved with various restriction endonucleases and subcloned to M13Δ*lac*182. Finally, we obtained large and clear plaques of the recombinant M13Δ*lac*182 carrying only the 229-nt *Dra*I-*Dde*I segment, named M13Δ*lac*182/229. The in vivo growth activity of M13Δ*lac*182/229 showed that this signal could recover the defect in replication activity of M13Δ*lac*182 (16).

To examine whether the *ssi* signal, which was primarily detected by the M13 phage system, is functional for replication of the plasmid itself, we constructed a mini-pGKV21 plasmid and a deletion mutant of the *ssi* signal on mini-pGKV21 DNA, named pWVAp and pWVAp/Δ*ssi*, respectively (Fig. 2). Colony sizes of *E. coli* cells harboring each of plasmids pWVAp and pWVAp/Δ*ssi* were compared on ampicillin-containing plates after overnight incubation at 37°C (Fig. 2). *E. coli* TG1[pWVAp] cells formed colonies larger than those of *E. coli* TG1[pWVAp/Δ*ssi*], indicating that the *ssi* signal is essential for replication activity or plasmid maintenance.

Replication activities of plasmids pWVAp and pWVAp/Δ*ssi* were examined by estimation of the amount of accumulated

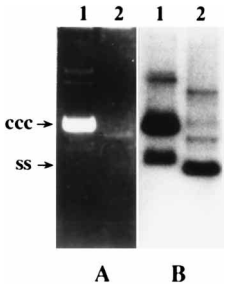


FIG. 3. Generation of plasmid ssDNAs from *E. coli* cells harboring either pWVAp or pWVAp/Δ*ssi*. Shown are 0.8% agarose gel electrophoresis (A) and Southern blot analysis (B) of plasmid DNAs prepared from *E. coli* harboring either pWVAp (lane 1) or pWVAp/Δ*ssi* (lane 2). Relative positions of covalently closed circular (ccc) and single-stranded (ss) DNAs are indicated.

plasmid ssDNA in *E. coli*. Total plasmid DNA was isolated from the same amounts of cells harboring pWVAp or pWVAp/Δ*ssi*. Each DNA preparation was subjected to agarose gel electrophoresis and, after denaturation and neutralization, to Southern hybridization. The results are shown in Fig. 3. With plasmid pWVAp, containing the *ssi* signal, plasmid ssDNA was approximately 20% as abundant as plasmid dsDNA, as determined by densitometric scanning of the autoradiograph presented in Fig. 3. However, deletion of the *ssi* signal on the plasmid resulted in accumulation of a high level of ssDNA and a greater reduction in relative plasmid copy number than that of plasmid pWVAp, indicating that in *E. coli*, this 229-nt *ssi* signal is functional for conversion of ssDNA to dsDNA in the plasmid itself.

The effect of the *ssi* signal on plasmid maintenance was examined with pWVAp and pWVAp/Δ*ssi* in *E. coli*. The results are shown in Fig. 4. Deletion of the *ssi* signal resulted in a low level of plasmid maintenance, about 5% of that in cells harboring plasmid pWVAp/Δ*ssi*, after 20 generations of growth. This result could be correlated to the differences of colony size and plasmid copy number between cells harboring either pWVAp or pWVAp/Δ*ssi*.

***E. coli* RNA polymerase directly synthesizes RNA primers on the *ssi* signal of pGKV21.** The minus origins of most ssDNA plasmids are known to require the host RNA polymerase for

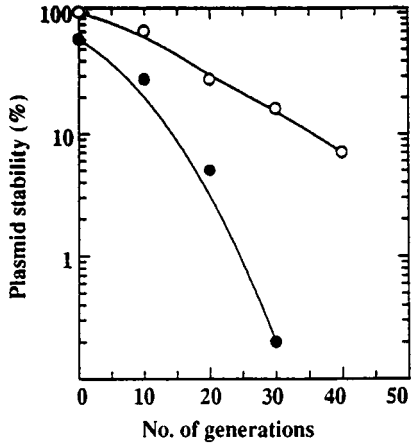


FIG. 4. Maintenance of plasmid pWVAp (○) or pWVAp/Δ*ssi* (●) in *E. coli*. Cells carrying one of the two plasmids were cultured during many generations in the absence of ampicillin. Plasmid stability was determined on ampicillin-containing agar plates by counting the antibiotic-resistant cells.

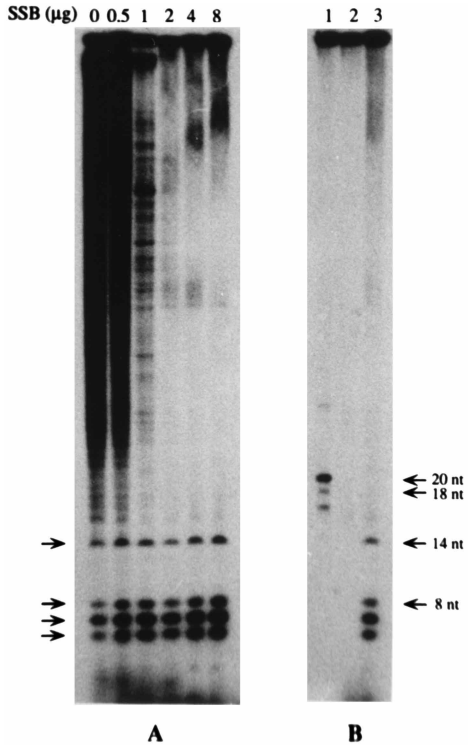


FIG. 5. Primer RNA synthesis on ssDNA template of M13Δ*lac*182/229. (A) Primer RNAs were synthesized on ssDNA template of M13Δ*lac*182/229 by adding *E. coli* RNA polymerase and increasing amounts (0 to 8 μg) of *E. coli* SSB protein. The products were analyzed on a 12% polyacrylamide–7 M urea gel. The arrows indicate the shorter RNAs considered to be the primer RNAs. (B) Primer RNAs were synthesized on three ssDNA templates of M13mp18 (lane 1), M13Δ*lac*182 (lane 2), and M13Δ*lac*182/229 (lane 3) by adding *E. coli* RNA polymerase and *E. coli* SSB protein.

conversion of ssDNA replication intermediates to the plasmid dsDNA form.

To determine the requirement of *E. coli* RNA polymerase for priming on the 229-nt *ssi* signal, we carried out an in vitro reaction for primer RNA synthesis, which consists of phage ssDNA template of M13Δ*lac*182/229, *E. coli* RNA polymerase, and various concentrations of *E. coli* SSB protein (Fig. 5A). In the absence of *E. coli* SSB protein, the synthesized RNAs were random in length. However, when the amounts of *E. coli* SSB protein were increased up to 8 μg, shorter RNAs considered to be the primer RNAs were more clearly visible.

To determine whether these shorter RNAs are the specific transcripts synthesized from the 229-nt *ssi* signal region, we carried out an in vitro primer RNA synthesis with three phage ssDNAs, M13mp18, M13Δ*lac*182, and M13Δ*lac*182/229 (Fig. 5B). M13mp18 DNA synthesized a primer RNA about 20 nt long, whereas M13Δ*lac*182 did not generate any detectable amount of primer RNA. In the case of M13Δ*lac*182/229, primer RNAs of discrete lengths were detected, as in Fig. 5A. The longest primer RNA synthesized was about 14 nt long. These results indicate that *E. coli* RNA polymerase directly recognizes the *ssi* signal and synthesizes primer RNAs dependent on the presence of *E. coli* SSB protein.

**Stem-loop I is essential for priming by *E. coli* RNA polymerase.** It has been shown that the *ssi* signals of *E. coli* plasmids and minus origins of ssDNA plasmids generally contain one or more stem-loop structures, as a prerequisite for the conversion of ssDNA to dsDNA. The *ssi* signal of pGKV21 contains two stem-loop structures based on its palindromic sequences (Fig. 1). Stem-loop I exhibits some characteristics of the minus origins of other ssDNA plasmids. That is, the consensus sequence, 5′-TAGCGT-3′, present in the loop of the *palA*-type minus origin, was also located in the loop of stem-loop I of the 229-nt *ssi* signal (9, 20). In addition, stem-loop I shows some sequence similarity with the complementary-strand origin of *E. coli* ssDNA phage ϕX174 (19, 20), but the roles that they play remain to be studied.

To analyze the effect of each of the two stem-loop structures within the *ssi* signal on the level of primer RNA synthesis, stem-loop I-deleted phages and stem-loop II-deleted phages were constructed from M13Δ*lac*182/229, and the activities of these templates for primer RNA synthesis in vitro were measured. The extent of each deletion is shown in Fig. 6A and 7, and the level of primer RNAs synthesized by *E. coli* RNA polymerase is shown in Fig. 6B. Stem-loop I-deleted phage Δ5-42 (from positions 42 to 229) was constructed by deletion of sequence within a part of the leftward stem of stem-loop I homologous to the consensus sequence, $RS_B$, identified as a site of cointegrate formation between small plasmids in gram-positive bacteria. Δ5-83 (from positions 83 to 229) retained the rightward stem and loop of stem-loop I containing 5′-TAGCGT-3′, a consensus sequence placed on the loop of the *palA*-type minus origin. Δ5-89 (from positions 89 to 229) lacked the 5′-TAGCGT-3′ sequence from Δ5-83. Δ5-153 (from positions 153 to 229) was constructed by deletion of all of stem-loop I. Stem-loop II-deleted phage Δ3-209 (from positions 1 to 209) retained the leftward stem and loop of stem-loop II. Δ3-183 (from positions 1 to 183) and Δ3-173 (from positions 1 to 173) lacked all of stem-loop II.

When deletion reached position 42 (Δ5-42) within stem-loop I, the amounts of the synthesized RNA primers were decreased to less than half of the amount of M13Δ*lac*182/229. In mutant Δ5-83, in which half of the sequence contributing to the formation of stem-loop I is deleted, its RNA primers were not synthesized as those of M13Δ*lac*182 didn't. These data indicate that the structural conformation of stem-loop I and/or primary sequences within this structure are essential for priming by *E. coli* RNA polymerase. On the other hand, in mutant Δ3-209, deleted of the rightward stem of stem-loop II, primer RNAs were still synthesized, albeit at a slightly reduced level. Two additional 3′-deletion mutants, Δ3-183 and Δ3-173, which lacked all of stem-loop II, produced primer RNAs at a level similar to that for Δ3-209.

In addition, to monitor the effects of deletion mutations of stem-loops I and II on SSI activity in vivo, growth of the mutant phages was measured. As shown in Table 1, the SSI activity of stem-loop I-deleted mutant Δ5-42 was greatly reduced but still slightly higher level than those of mutants Δ5-83 and Δ5-89. Extension of the deletion to position 83 (Δ5-83) resulted in nearly the same level as that of M13Δ*lac*182. These results are in good agreement with those of in vitro primer RNA synthesis as shown in Fig. 6B. Stem-loop II-deleted mutants also grew more poorly than M13Δ*lac*182/229 but significantly better than M13Δ*lac*182 and stem-loop I-deleted mutants. Thus, it seems that stem-loop II is also required for full SSI activity.

**Determination of the critical region within the *ssi* signal.** By performing random mutagenesis via a modified PCR, we tried to identify the critical region within the *ssi* signal which contributes to SSI activity. We obtained 21 mutants containing nucleotide substitutions. The positions of the nucleotide substitutions were scattered all over *ssi* signal as shown in Fig. 7. A48G, A98G, T113C, and A125T, located mainly on the bulge structure of stem-loop I, showed slight or no effect on SSI activity. However, mutants T21CT78CT106C (consisting of

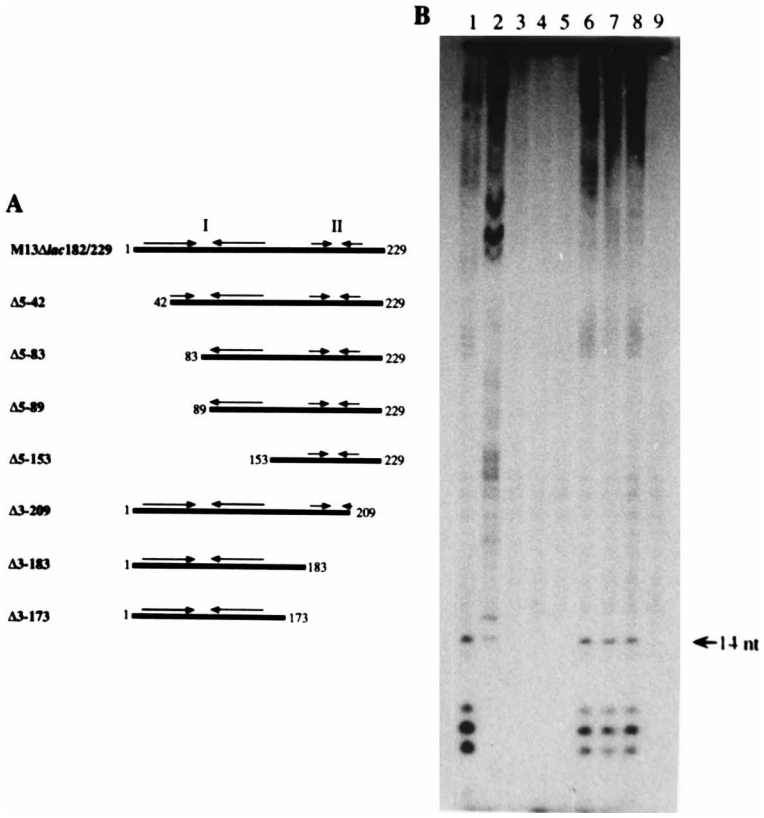


FIG. 6. Primer RNA synthesis in vitro, using deletion derivatives of M13Δ*lac*182/229. (A) Schematic representation of the *ssi* signal after deletion of stem-loop I or stem-loop II. The nucleotide sequences of deletion mutants are presented in Fig. 7. Two stem-loop structures are indicated by I and II. (B) Generation of the primer RNAs by deletion derivatives. Primer RNAs were synthesized on ssDNA templates by *E. coli* RNA polymerase. Template DNAs used: lane 1, M13Δ*lac*182/229; lane 2, Δ5-42; lane 3, Δ5-83; lane 4, Δ5-89; lane 5, Δ5-153; lane 6, Δ3-209; lane 7, Δ3-183; lane 8, Δ3-173; lane 9, M13Δ*lac*182.

T21C, T78C, and T106C), T27CA143G (consisting of T27C and A143G), A40TA139G (consisting of A40T and A139G), and A105GT149C (consisting of A105G and T149C), which had substitutions at two or three nucleotides on stem-loop I, showed a great reduction in SSI activity. Most of these nucleotides are positioned on the stem of stem-loop I, suggesting that they may be essential for formation of a stem-loop structure and directly involved in recognition by *E. coli* RNA polymerase. Three mutants, T168C, A181G, and A188G, which had substitutions of only a single nucleotide on the internal flanking region between stem-loops I and II, also showed a great reduction in SSI activity, suggesting that this internal flanking region between stem-loops I and II is also important for maximal SSI activity.

## DISCUSSION

Plasmid pGKV21 was derived from the lactococcal plasmid pWV01, originally isolated from *Lactococcus lactis* subsp. *cremoris* Wg2 and shown to be able to replicate in gram-positive as well as gram-negative bacteria, including *E. coli* (18, 33). Based on the nucleotide sequence similarities of the leading-strand replication region, plasmid pWV01 belongs to the pE194 group of rolling-circle plasmids (6, 10, 20).

In this study, to understand the replication mechanism of pGKV21 as a broad-host-range plasmid, we investigated the functional features of the *ssi* signal of plasmid pGKV21 in *E. coli*. To investigate whether the *ssi* signal of pGKV21 can function in *E. coli*, we used a mutant M13 phage vector (M13Δ*lac*182) which is defective in the origin function for minus-strand synthesis and forms very small and turbid plaques. This vector was originally constructed for screening of *ssi* signals from *E. coli* plasmids and phages.

We have previously showed that an *ssi* signal of pGKV21 is situated at the 229-nt *Dde*I-*Dra*I fragment (16). The in vivo growth activity of the mutant M13 phage, M13Δ*lac*182, was partially restored by insertion of this DNA fragment carrying the 229-nt *ssi* signal. However, the replication activity of the *ssi* signal in pGKV21 was lower than those of *ssi* signals of *E. coli* plasmids. It seems that in *E. coli*, priming efficiency of the *ssi* signal isolated from gram-positive bacterial plasmid pGKV21 may be lower than those of *E. coli* plasmids.

The plasmid (pWVAp/Δ*ssi*) lacking an *ssi* signal accumulated much more plasmid ssDNA than the wild-type plasmid (pWVAp) did. Moreover, deletion of this region reduces the plasmid copy number and causes plasmid instability, suggesting that in *E. coli*, this *ssi* signal directs its lagging-strand synthesis as a minus origin of plasmid pGKV21.

Most minus origins of ssDNA plasmids have been characterized as host specific, except for the minus origins of pUB110 and pBAA1 (10, 31). For example, a *palA* present on the minus origins of numerous staphylococcal plasmids is not recognized in *Bacillus subtilis* (9). The minus origin of streptococcal plasmid pLS1 is efficiently recognized only in *Streptococcus pneumoniae* (5). Thus, nearly all minus origins seem to confer host specificity. In this work, we showed that the *ssi* signal of pGKV21, which was derived from *L. lactis*, is also functional in

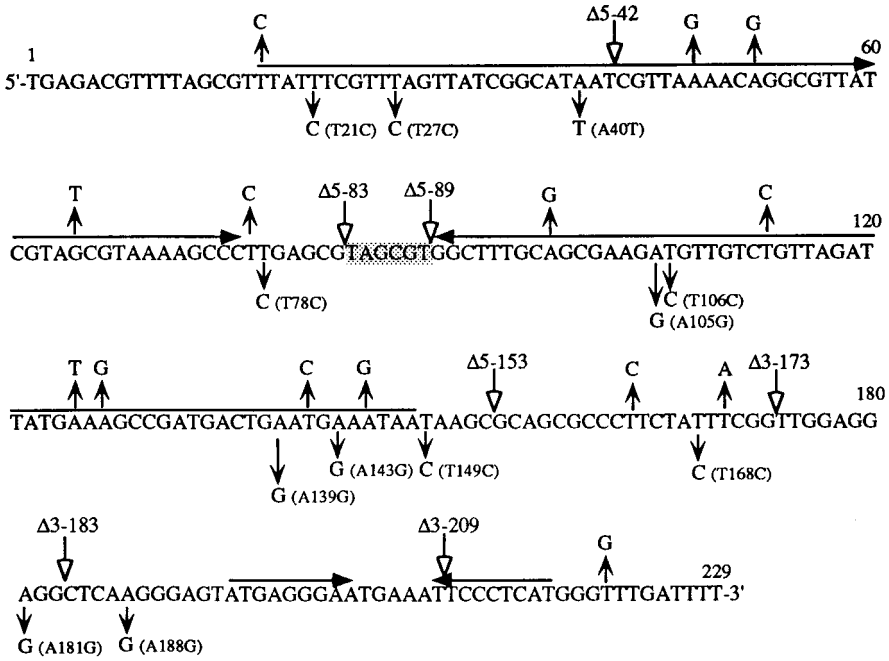


FIG. 7. Nucleotide sequence and mutated positions of the *ssi* signal. The nucleotide sequence of the 229-nt *ssi* signal is presented. Nucleotide numbering is the same as Fig. 1. Upward arrows indicate mutations that had little or no effect on SSI activity; downward arrows indicate mutations that blocked SSI activity. The endpoints of deletion are indicated by open arrowheads above the nucleotide sequence. A shaded box indicates the consensus sequence presented in the loop of *palA*-type minus origins.

*E. coli* as a minus origin for conversion of the ssDNA intermediate to the plasmid dsDNA form.

The minus origins of most ssDNA plasmids are known to require the host RNA polymerase for conversion of the ssDNA intermediate to the plasmid dsDNA form. As described in this report, *E. coli* RNA polymerase is involved in synthesis of RNA primers on the *ssi* signal of pGKV21 dependent on the presence of *E. coli* SSB protein. This *ssi* signal contains two potential stem-loop structures, stem-loops I and II. Stem-loop I showed some nucleotide sequence homologies with other previously described *palA*-type minus origins, as well as with the ssDNA-to-dsDNA conversion signal of the *E. coli* ssDNA phage ϕX174. The ϕX174-type conversion mechanism uses the primosome complex for complementary-strand synthesis, and the RNA primer is synthesized by primase, whereas our data indicate that *E. coli* RNA polymerase is used by the pGKV21 *ssi* signal. Therefore, the pGKV21 *ssi* signal, although sharing homologous sequence motifs with the ϕX174-type primosome assembly site, may function at a priming stage by a different mechanism in *E. coli.*

TABLE 1. Growth of deletion mutants and controls

| Phage | Sequence (nt) | Phage titer[a] (PFU/ml) | Index of SSI function[b] |
|---|---|---|---|
| M13Δ*lac*182/229 | 1–229 | $2.4 \times 10^{11}$ | $7.7 \times 10^{1}$ |
| Δ5-42 | 42–229 | $2.9 \times 10^{10}$ | $2.1 \times 10^{0}$ |
| Δ5-83 | 83–229 | $2.0 \times 10^{10}$ | $1.4 \times 10^{0}$ |
| Δ5-89 | 89–229 | $2.1 \times 10^{10}$ | $1.5 \times 10^{0}$ |
| Δ5-153 | 153–229 | $1.6 \times 10^{10}$ | $1.1 \times 10^{0}$ |
| Δ3-209 | 1–209 | $5.1 \times 10^{10}$ | $3.6 \times 10^{0}$ |
| Δ3-183 | 1–183 | $4.8 \times 10^{10}$ | $3.4 \times 10^{0}$ |
| Δ3-173 | 1–173 | $4.5 \times 10^{10}$ | $3.2 \times 10^{0}$ |
| M13Δ*lac*182 | | $1.4 \times 10^{10}$ | 1 |

[a] At 8 h after infection, measured as described in Materials and Methods.
[b] Calculated from the phage titer for each recombinant phage at 8 h as follows: PFU of recombinant phage per milliliter/PFU of M13Δ*lac*182 per milliliter.

The importance of two stem-loop structures is demonstrated by deletion and point mutation analyses of the *ssi* signal of pGKV21. Nested deletion of sequences within the stem-loop I structure leads to the abolition of both in vivo SSI activity and in vitro primer RNA synthesis by *E. coli* RNA polymerase. In the random mutagenesis analysis, some inactivating mutation (with a single nucleotide substitution) was in stem-loop I structure, but these nucleotides are mainly distributed on the bulge structure. All nucleotides which showed the destruction of SSI activity were positioned on the stem of stem-loop I. These results indicate that the conformation and/or sequences of stem-loop I are very important for primer RNA synthesis and SSI activity. Deletion of stem-loop II leads to a reduction in SSI activity and primer RNA synthesis, although their activities are higher than those of stem-loop I-deleted mutants. The single nucleotide substitution mutants, T168C, A181G, and A188G, on the internal flanking region between stem-loops I and II also showed a reduction in SSI activity, indicating that nucleotides of stem-loop II and internal flanking region between stem-loops I and II are required for maximal SSI activity.

Seegers et al. (30) have reported that in *L. lactis*, the functional pWV01 minus origin is located on a 294-nt *Cla*I–*Eco*47-3 fragment and consists of stem-loop I and II structures as described in this study. However, they described that in *L. lactis*, stem-loop I was shown to have a partial, RNA polymerase-independent activity for complementary-strand synthesis. In addition, they reported that stem-loop II, which had no activity of its own, was required for full minus-origin activity in an RNA polymerase-dependent manner through observation of the effects of rifampin on ssDNA-to-dsDNA conversion.

In the present report, we have suggested that 229-nt *ssi* signal of pGKV21, containing two stem-loop structures, directs its lagging-strand synthesis in *E. coli* as a minus origin of plasmid pGKV21. *E. coli* RNA polymerase directly recognizes the 229-nt *ssi* signal and synthesizes primer RNA dependent on the presence of *E. coli* SSB protein. In addition, stem-loop I is essential for priming by *E. coli* RNA polymerase on the SSB-coated ssDNA template, as concluded from the fact that nested deletion of sequences within the stem-loop I portion led to the abolition of both in vitro primer RNA synthesis and in vivo SSI activity.

Judging from the in vitro primer RNA synthesis and in vivo SSI activity described here, we assume that the replication of pGKV21 in *E. coli* may be regulated by a mechanism slightly different from that in gram-positive bacteria. However, determination of the precise mechanism for the initiation of lagging-strand synthesis requires further investigation.

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