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Functional characterization of the *repA* replication gene of linear plasmid prophage N15

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Abstract

The prophage of coliphage N15 is not integrated into the chromosome, but exists as a linear plasmid molecule with covalently closed ends. The only phage gene required for replication of circular N15 miniplasmids is *repA* (gene *37*). Here we show that RepA-driven replication of the N15-based circular and linear miniplasmids is independent of host DnaB helicase protein, but requires the host DnaG primase. Replication of phage N15 DNA during lytic growth following infection does not depend on either DnaG or DnaB, but DnaG is required for lytic development after induction of the N15 lysogen. Finally, protein sequence analysis and replication data using different mutant strains suggest that RepA protein combines helicase and primase functions.

Keywords: Linear plasmid; Replication; Primase; Bacteriophage

1. Introduction

The temperate phage N15 of *Escherichia coli* belongs to the lambdoid phage family. However, unlike lambda, the N15 prophage is not integrated into the chromosome [20], but is maintained as a linear plasmid with covalently closed hairpin ends [26]. This topology arises from a two-step mechanism (reviewed in [22]): first, the injected DNA is circularized via annealing of its cohesive ends; then a special phage-encoded enzyme, protelomerase (TelN), cleaves the circle at another site, *telRL*, and forms hairpin ends (telomeres). This cleavage-joining activity of purified TelN protein has been demonstrated in vitro [5].

Phage N15 is a useful model for studying the mechanism of replication of linear DNA with covalently closed ends. Such replicons are generally of eukaryotic origin, but a few bacterial examples are known: linear plasmids and chromosomes of *Borrelia* [3,8], the phage-plasmids ϕKO2 in *Klebsiella oxytoca* [4,24] and PY54 in *Yersinia enterocolitica* [12], and one of the two chromosomes of *Agrobacterium tumefaciens* [1,10]. We had demonstrated previously that N15 protelomerase is necessary for replication of the linear plasmid prophage through its action as a telomere-resolving enzyme; a deficiency in protelomerase results in the accumulation of circular head-to-head dimer intermediates [18]. In the current model of N15 plasmid replication (reviewed in [15]), replication is initiated at the *ori* site located in the left half of the prophage, within gene *37* (*repA*) [16]. Bidirectional replication from this asymmetrically located internal *ori* site results in duplication of the left telomere, which is then cut by protelomerase, leading to formation of a Y-shaped structure. After replication of the right telomere and subsequent processing, two linear molecules are produced [16].

Some information on the replication origin and requirements for its activity is available. The minimal plasmid replicon, able to drive replication of the circular N15 miniplasmid, is located within a 4-kb-long fragment that contains only the phage gene *repA* [16]. The minimal linear plasmid contains the telomeres and the protelomerase gene as well. To be maintained at a normal (low) copy number, the

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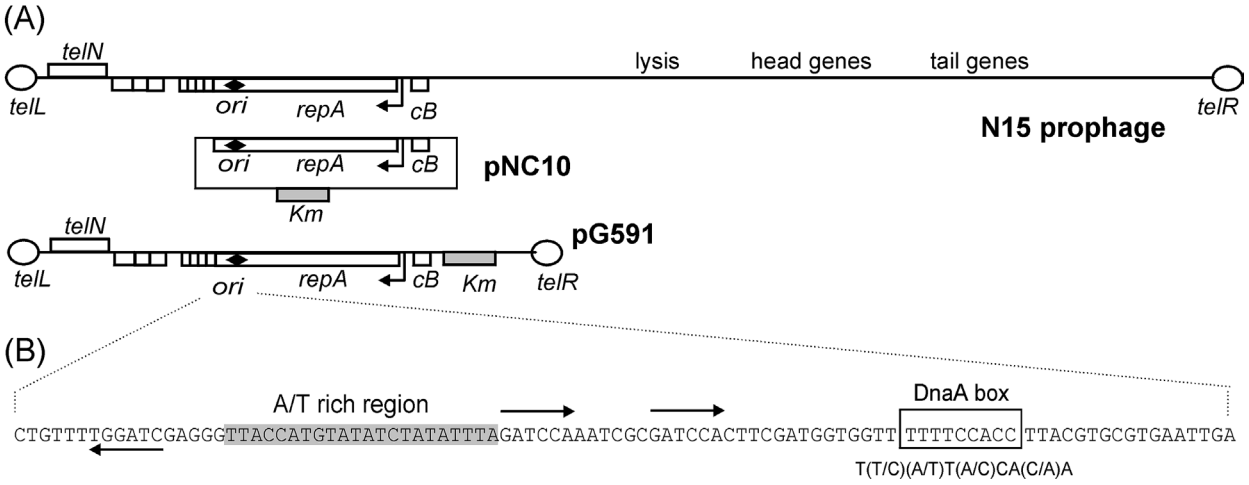

Fig. 1. (A) Maps of N15 plasmid prophage and miniplasmids based on the N15 replicon. Rectangles above and below the main line represent genes transcribed rightward and leftward, respectively. The promoter–operator site of *repA* is shown as a bent arrow. *ori*, N15 replication initiation site; *telN*, protelomerase gene; *cB*, gene, encoding the main phage repressor CB; *Km*, kanamycin resistance gene; *telL* and *telR*, left and right hairpin ends of the prophage created by protelomerase. The maps are not drawn to scale. (B) Nucleotide sequence of the replication origin. Thick arrows indicate GATCCA repeats and the A/T-rich region is shaded. The putative DnaA binding site is boxed, and the consensus sequence is shown below.

linear and circular miniplasmids must also carry the primary repressor gene, *39* (*cB*). The replication initiation region (Fig. 1) contains three GATCCA repeats arranged in an iteron-like manner, the sequence TTTTCCACC which is very similar to the *E. coli* consensus DnaA-binding site T(T/C)(A/T)T(A/C)CA(C/A)A [9], and a 22-bp A/T-rich region (18.2% G + C) located between two of the repeats [16]. N15 replication is found to be independent of *polA*, *recA* [27] and the heat shock genes *dnaJ*, *dnaK* and *grpE* [28], but shows a strong requirement for Dam methylation [22], which is consistent with the presence of the GATC Dam-methylase recognition sites in the putative iterons.

Sequence analysis of the RepA revealed that it is a homologue of primases of conjugative plasmids and of the phage P4 primase [21,22]. The latter enzyme, phage P4α replication protein, has been shown to have primase, helicase and origin recognition activity [31]. The importance of *repA* for replication of N15 DNA is further supported by the observation that all replication mutations isolated so far have been mapped to this gene [22].

In the present manuscript we analyze the amino acid sequence of RepA and investigate the dependence of replication of phage N15 and of N15 miniplasmids on host replication functions.

2. Materials and methods

2.1. Media, bacterial strains, bacteriophages and plasmids

Bacteria were grown in LB liquid medium or on LB agar plates at 37 °C unless otherwise indicated. Antibiotics were added as appropriate, at the following concentrations: ampicillin, 100 μg/ml and kanamycin, 50 μg/ml.

The prototrophic *E. coli* C [23] strain was used as the host for phage propagation, and DH10B [11] for maintenance of plasmids. Strain C-5582 (*dnaG3ts*) [29] was kindly provided by Gianni Dehò, GC2037 (*dnaB70ts*, *thr1*, *leuB6*, *hisG4*, *proA2*, *arg*$^+$, *ara*, *lacY*, *galK*, *xyl*, *mtl*, *F*$^-$) and LN3 (*dnaG308ts*)—by David Lane, and Ax727 (*dnaX2016ts*, *lac*$^-$, *rpsl*$^-$, *thi*$^-$) [7]—by Erich Lanka. Strains C-5582, GC2037 and Ax727 were grown at 30 °C.

The clear plaque mutant of bacteriophage N15, N15*cl6* has been described in [19]. The N15-based circular plasmid pNC10 consists of an N15 DNA fragment containing the *repA* and *cB* genes and a kanamycin resistance gene [17]. Linear plasmid pG591 [16] contains all N15 genes required for replication and maintenance in a linear form. Plasmid pCA12 [19], based on the pBR322 replicon, carries the N15 antirepressor gene (*ant*A) under the control of the arabinose-inducible $araP_{BAD}$ promoter.

2.2. Infection with phage N15

Bacterial cultures were grown in LB broth with shaking at 30 °C to 1–2 × 10^8 cells/ml. The cells were collected by centrifugation and resuspended in the N15 adsorption buffer (10 mM Tris (pH 7.7), 10 mM $MgCl_2$, 1 mM $CaCl_2$). After 30-min incubation at 30 °C with shaking, the phage N15 clear plaque mutant N15*cl9* was added at a multiplicity of infection of 10. Adsorption was allowed for 30 min at 30 °C without shaking. The bacteria were then pelleted by centrifugation and washed twice with the N15 adsorption buffer. The washing steps reduced the titer of unabsorbed phage to a level below 10^4 phages/ml. Finally, the pellet was resuspended in prewarmed (to 30 or 42 °C) LB broth ($t = 0$) and further incubated at 30 or 42 °C with shaking. The numbers of infected centers ("yielders") were assayed 20 min after the start of lytic development by plating the infected cells on an

E. coli C lawn (note that under these conditions only cells in which infection follows the lytic pathway produce plaques). Titers of the progeny phages were assayed 2 h after the start of infection.

3. Results

3.1. Analysis of the amino acid sequence of the RepA protein

RepA is a large protein with a calculated molecular weight of 149 kDa (1324 aa). Analysis of the amino acid sequence of RepA revealed several characteristic motifs (Fig. 2). A region of about 100 aa located in the N-terminal half of the protein showed significant sequence similarity to plasmid-encoded DNA primases, particularly the phage P4α replication protein (approximately 30% identity and 43% similarity in a 102-aa fragment). In particular, RepA contained sequence EGFATG similar to the EGYATA motif that is characteristic of prokaryotic primases and has been shown by mutational analysis to be essential for the primase activity of the P4α replication protein [25]. Another characteristic motif, a potential Mg^{2+} binding site found in various bacterial polymerases [2], was also present in RepA (DxD, Fig. 2). The distance between these two motifs was similar to that in other primases [25].

Another domain of RepA was very similar to the Walker A Box nucleotide binding site [30] found in DNA helicases, such as the P4α protein, gp41 of phage T4, and *E. coli* proteins UvrA, Rep, and UvrD [25]. The second motif involved in NTP hydrolysis, the Walker B Box, was also present in RepA.

A search for motifs relevant to DNA recognition produced two results. First, the region spanning amino acids 24–45 was predicted by the Dodd and Egan algorithm [6] (SD score 3.05) to form a DNA binding helix-turn-helix motif (Fig. 2). Second, regions of homology to different bacterial and eukaryotic proteins known to have helicase and origin-recognition activity were found in the C-terminal part of RepA. In particular, a region from aa 714 to aa 856 showed a pronounced homology to the origin binding helicase protein of the African swine fever virus (28% identity and 47% similarity, respectively, in the 143 aa region).

3.2. Replication of N15-based miniplasmids in E. coli *strains carrying mutations affecting replication functions*

The N15-based circular plasmid pNC10 consists of an N15 DNA fragment containing the *repA* and *cB* genes and a kanamycin resistance gene. Due to the presence of the *cB* repressor gene, this plasmid is maintained at low copy number; its replication can be stimulated by inactivation of the repressor using, for instance, the N15 antirepressor AntA. Replication of pNC10 in this case could be easily monitored since its copy number increases more than 10-fold. The plasmid pCA12, which carries the *antA* gene under the control of the arabinose-inducible $araP_{BAD}$ promoter, and pNC10 were introduced into *E. coli* strains carrying temperature sensitivity mutations (30 °C permissive temperature, 42 °C non-permissive temperature) in genes essential for replication. The following strains were used: C-5582 (*dnaG3*, primase), GC2037 (*dnaB70*, helicase), Ax727 (*dnaX2016*, DNA polymerase III subunit), and the control strain *E. coli* C.

Cultures growing at 30 °C were divided into two parts; one was shifted to 42 °C, the other left at 30 °C; 5 min later, replication of pNC10 was activated by adding arabinose to the medium to 0.1%, and incubation of the cultures was continued for two more hours. Plasmid DNA was then isolated using an alkali lysis method, digested with appropriate restriction enzymes, and analyzed by agarose gel electrophoresis. Experiments with strain C-5582 were performed at four different temperatures, 30, 35, 40 and 42 °C. The increase in the intensity of the pNC10-specific band compared with the pCA12-specific band demonstrated active replication of pNC10. Shifting the temperature from 30 to 42 °C resulted in immediate arrest of chromosomal DNA synthesis in the case of strains GC2037 and Ax727 (quantitative determination of total DNA amount, data not shown). In the case of strain C5582, the amount of chromosomal DNA continued to increase during one generation, and synthesis then stopped. This was the expected result, since *dnaG3* is a so-called “slow stop” *dnaG* mutation resulting in deficiency of replication initiation [13].

The results (Fig. 3) show that pNC10 replication was not affected by the mutation in *dnaB* helicase (strain GC-2037). In this case, the increase in the pNC10 copy number after activation of replication was lower than with the control strain *E. coli C*, presumably due to a difference in genetic background, but it was not affected by a mutant allele of the host DNA helicase, *dnaB70* (compare lanes 5 and 6, Fig. 3). However, pNC10 did not replicate at the non-permissive temperature in strain Ax727 in which the mutation *dnaX2016* rendered one of the DNA polymerase III subunits temperature-sensitive, indicating the dependence of N15 replication on the host DNA polymerase. This result was expected, since we did not find any homology between RepA and DNA polymerases. In subsequent experiments strain Ax727 was used as a negative control. The most interesting and unexpected result of this experiment was that pNC10 replication depended on the host DnaG primase (strain C-5582).

To verify these results, we carried out several additional experiments. We showed (Fig. 4) that replication of the linear N15-based plasmid, pG591 also depended on *dnaG* (C-5582), but not on *dnaB* (GC2037) or *dnaX* (Ax727). We confirmed the dependence of pG591 replication on *dnaG* using Southern blot analysis of total cellular DNA rather than analysis of plasmid DNA in minipreps to exclude the possibility of selective loss of particular forms of plasmid DNA during extraction. We also showed that another *ts dnaG* al-

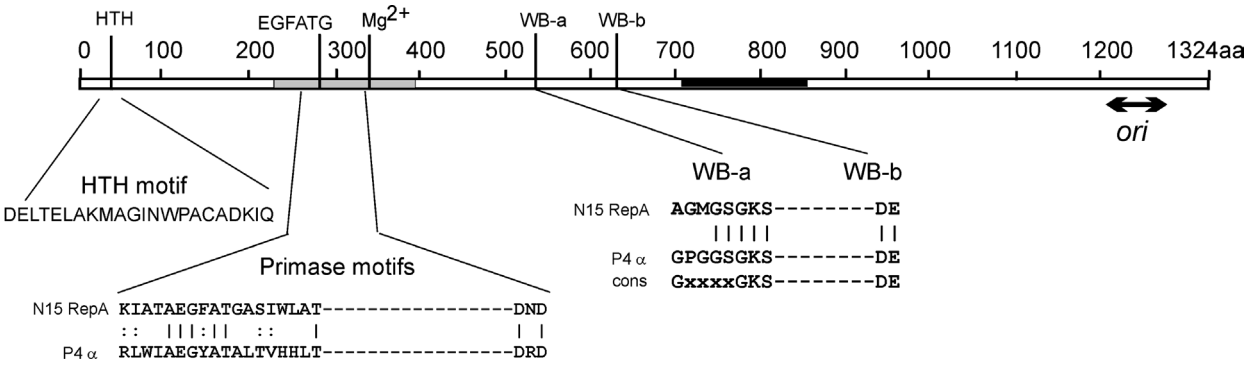

Fig. 2. Structure of RepA: arrangement of sequence motifs within the amino acid sequence of the protein. The bar represents RepA, the shaded segment shows the region exhibiting amino acid similarities between RepA and primases of IncI/IncP plasmids and P4α protein. The black region shows the region of homology between RepA and origin-binding proteins of African swine fever virus and human herpesvirus. Locations of the EGFATG motif, the DxD motif (Mg^{2+}) and Walker boxes A and B (WB-a and WB-b) are indicated. Comparisons of amino acid sequences of N15 RepA and P4α proteins in these regions and consensus sequences of WB-a and WB-b [30] are shown beneath. HTH, putative helix-turn-helix motif; *ori*, the region of RepA which corresponds to the position of the N15 *ori* site within *repA* gene.

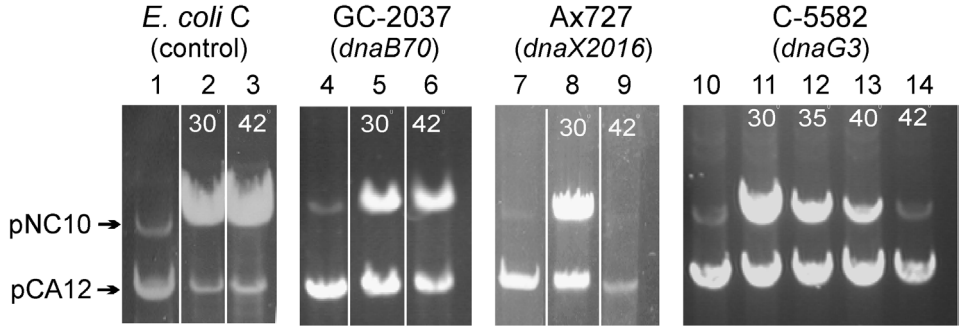

Fig. 3. Replication of the circular N15-based miniplasmid pNC10 in *E. coli* strains carrying *ts* mutations in replication genes. Plasmid DNAs from *E. coli* strains carrying plasmids pCA12 and pNC10 were isolated, digested with *Eco*RV and analyzed by agarose gel electrophoresis. The restriction enzyme *Eco*RV cut plasmids pCA12 and pNC10 once. Lanes 1, 4, 7, 10: DNA isolated before activation of replication of pNC10 (see details in text). Other lanes: DNA isolated 2 h after activation of replication. Induction was carried out at different temperatures as indicated.

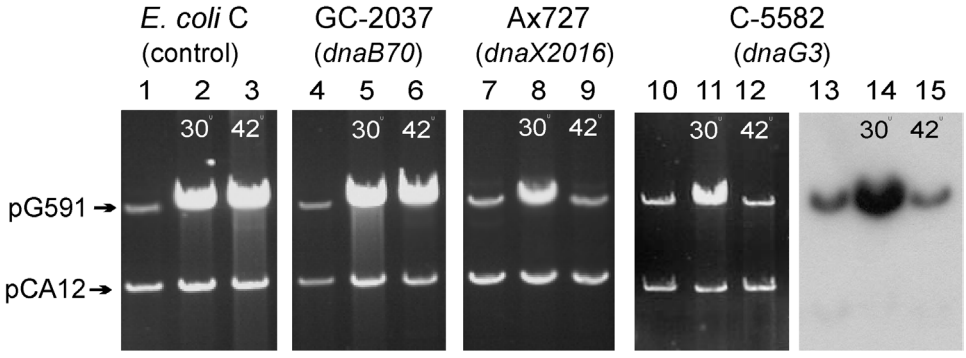

Fig. 4. Replication of the linear N15-based miniplasmid pG591 in *E. coli* strains carrying *ts* mutations in replication genes. Plasmid (lanes 1–12) or total (lanes 13–15) DNAs from *E. coli* strains carrying plasmids pCA12 and pG591 were isolated, digested with *Nhe*I and analyzed by agarose gel electrophoresis (lanes 1–12) or Southern blot (lanes 13–15). The restriction enzyme *Nhe*I cut pCA12 once, and did not cut the linear plasmid pG591. Lanes 1, 4, 7, 10 and 13: DNA isolated before activation of replication of pG591 (see details in the text). Other lanes: DNA isolated 2 h after activation of replication. Induction was carried out at permissive temperature, 30 °C, or at non-permissive temperature, 42 °C, as indicated.

Table 1
Influence of host mutations affecting replication functions on N15 lytic development

Host strain	Mutation	Function affected	Burst size[a]	
			30 °C	42 °C
E. coli C	–	–	124	72
C-5582	*dnaG3*	DnaG, primase	105	81
GC2037	*dnaB70*	DnaB, helicase	20	19
Ax727	*dnaX2016*	PolIII subunit, polymerase	33	< 0.3

[a] Burst size = progeny phages/yielders. Clear-plaque mutant N15*cl6* was used for infection.

lele (*dnaG308*) prevented pNC10 and pG591 replication at the non-permissive temperature (data not shown).

3.3. Phage N15 lytic growth in host strains carrying mutations affecting replication functions

The above data show that replication of a circular N15-based miniplasmid was independent of *dnaB* but depended on *dnaG*. It is known that the same replicon is involved in phage N15 replication in the course of lytic development [16]. However, the requirements for host functions might be different in this case. We therefore analyzed the outcome of infection by an N15 clear-plaque mutant (N15*cl6*) of host strains carrying mutations in the *dnaG* and *dnaB* genes.

The efficiency of infection of *E. coli* C, C-5582 and GC 2037 by phage N15 at 30 and 42 °C was determined quantitatively by measurement of burst size (as described in [19]). As seen in Table 1, mutation in DNA primase (*dnaG3)*, but not in DNA polymerase III (*dnaX2016*), did not affect N15 development at the non-permissive temperature. In the case of strain GC2037, the N15 burst size was lower than with the control strain *E. coli* C, presumably due to a difference in genetic background, but it was not affected by mutation in host DNA helicase, *dnaB70* (cf. burst sizes at 30 and 42 °C, Table 1). These results show that N15 lytic development, and replication in particular, did not depend on DnaB nor, in contrast to replication of miniplasmids pNC10 and pG591, on DnaG (Table 1).

3.4. Dependence of phage N15 DNA replication on host dnaG

The above results indicated that phage N15 DNA replication was independent of *dnaG* in the case of infection. To further verify this result, we directly monitored the efficiency of N15 DNA synthesis using Southern blot analysis of total cellular DNA isolated from N15-infected C-5582 cells. The results (Fig. 5) showed that N15 DNA replication in this case proceeded equally well at 30 and at 42 °C.

We tested whether phage N15 DNA replication was also independent of *dnaG* when lytic development was initiated by prophage induction. Replication of N15 prophage in C-5582(N15)/pCA12 cells was activated by the N15 antirepressor, as in the experiments with pNC10. Plasmid DNA was isolated from cultures incubated with arabinose at 30 and 42 °C and analyzed by agarose gel electrophoresis. The results clearly showed that in this case N15 DNA replication depended on *dnaG*: the plasmid copy number increased at 30 °C, but not at 42 °C, while in the control strain, *E. coli* C (N15)/pCA12, N15 replicated equally at both temperatures (Fig. 5). Activation of lytic development resulted in no detectable progeny phage production in C-5582 (N15)/pCA12 cells (less then 0.1 phage per cell) at 42 °C, whereas after 3 h at 30 °C, phage production, 50–70 per cell, was as high as in *E. coli* C (N15)/pCA12 (data not shown). Thus, replication of phage N15 DNA depended on *dnaG* if it was activated in the prophage state, but did not require host primase in the case of phage infection.

4. Discussion

4.1. Proposed functions of the RepA protein

Identification and characterization of proteins involved in replication represent one of the principal steps in developing a model of replication. Several lines of evidence suggest the existence of a primase activity of RepA. First, sequence analysis has revealed two highly conserved motifs, EGYATA and DxD, specific for bacterial replication proteins having primase activity. We did not, however, find the third motif, a proposed Zn^{+} binding region containing CxxC clusters, which is present in the P4α protein [25] and phage T7 gp4, where this motif is required for recognition of the priming sites [14]. The EGYATA motif is important for P4α protein function because the E to Q and T to S substitutions disrupt its primase function [25]. The importance of the corresponding N15 RepA sequence EGFATG for activity of the N15 RepA is supported by an observation that the F to S mutation in this motif results in temperature-sensitive replication of N15 (mutation *ts52*, [16]). Secondly, lytic phage development is independent of the host DnaG primase.

Similar experiments showed that N15 replication is independent of bacterial helicase DnaB, implying that N15 RepA has helicase activity. In this case, both phage N15 lytic development and replication of N15-based circular and linear miniplasmids containing *repA* are independent of the host DnaB helicase.

The independence of N15 replication from the bacterial helicase (DnaB) and, in the case of infection, from primase (DnaG) does not necessarily mean that RepA has primase and helicase activities. However, considered together with the presence of motifs characteristic of bacterial primases and helicases in the amino acid sequence of RepA, these data suggest that RepA is a multifunctional protein with primase and helicase activities.

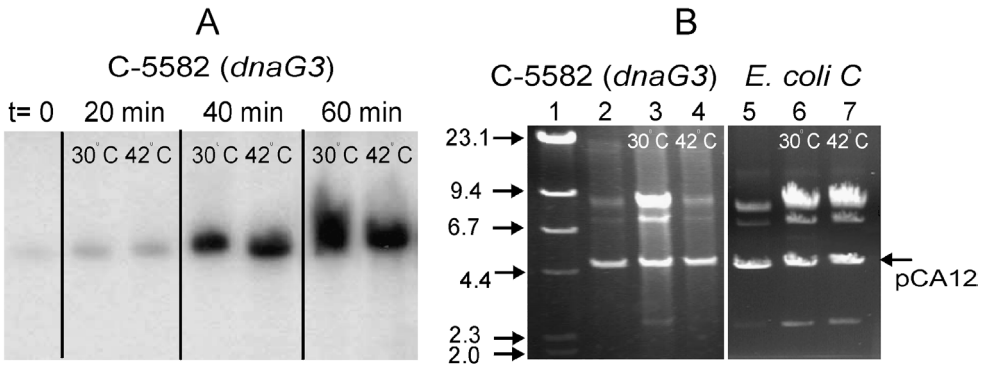

Fig. 5. (A) Replication of phage N15 DNA in the course of infection of C-5582 cells at 30 and 42 °C. Total DNA was isolated at different times (shown in minutes) after the start of infection and analyzed by Southern blot using an N15-specific radiolabeled probe. (B) Replication of phage N15 DNA after activation of lytic development in the lysogen. Plasmid DNAs from strains C-5582 (lanes 2–4) and *E. coli* C (lanes 5–7) carrying prophages N15 and pCA12 were isolated, digested with *Eco*RI and analyzed by agarose gel electrophoresis. Lane 1: molecular weight marker; arrows indicate position and sizes (in kb) of fragments. Lanes 2 and 5: DNA isolated before activation of lytic development. Lanes 3 and 6: DNA isolated from the same strains as in lanes 2 and 5 but at 1 h after activation of lytic development. The experiment was carried out at 30 °C. Lanes 4 and 7: the same as in lanes 3 and 6, but the experiment was carried out at 42 °C. Position of pCA12 is shown by arrow; other bands resulted from digestion of N15 DNA with *Eco*RI (9.07; 8.83; 8.77; 8,48; 7.32; 2.62; 0.74; 0.56 kb).

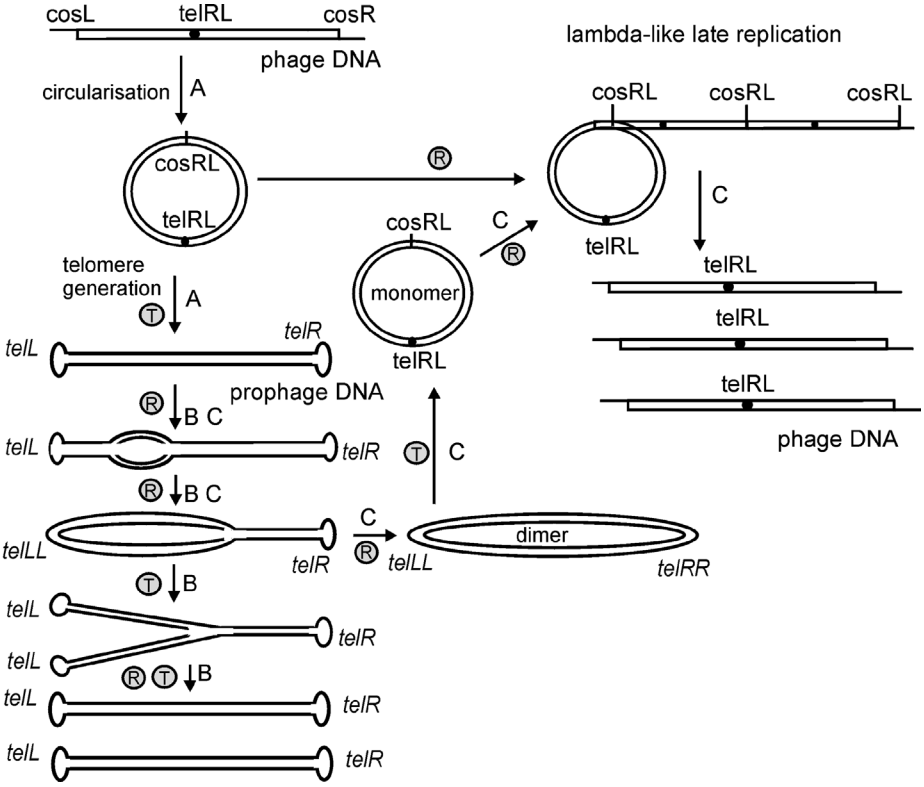

Fig. 6. Possible mechanisms of interconversions of different forms of N15 DNA and its replication in the course of lytic development. (A) Conversion of infecting phage DNA to a linear plasmid. (B) Replication of N15 plasmid prophage. (C) Replication of N15 DNA in the case of activation of lytic development in the lysogenic culture. Mechanisms of conversion of linear plasmid into a circular monomer is unknown, but we suggest that at some step of lytic development replication of the prophage could result in formation of a circular head-to-head dimer that could then be processed by protelomerase into two circular monomers. The known or suggested participation of the N15 genes TelN (T) and RepA (R) at the individual steps is shown.

4.2. Involvement of the host DnaG primase in replication of N15 DNA

One of the most interesting results of this work is the observation that DnaG primase is not required for N15 replication following infection, but is required for replication of circular and linear miniplasmids and following prophage induction. The reason for this difference might reflect differences in the mechanisms of plasmid prophage replication and replication of N15 DNA in the course of infection. Bidi-

rectional replication of the prophage is initiated at the *ori* site located within *repA*, and results in duplication of the left telomere, which is then cut by protelomerase, leading to formation of a Y-shaped structure. After replication of the right telomere and subsequent cutting, two linear molecules are produced. Our results suggest that host DnaG primase is required for this replication pathway, as well as for replication of a circular N15-based miniplasmid, which is believed to proceed following the standard theta-mode.

The mechanism of N15 DNA replication during lytic development is less well known but it is very likely, in view of the strong similarity of N15 morphogenetic genes to those of phage lambda [21], that at some step of lytic growth the phage DNA molecule is converted to a circular "head-to-tail" monomer, and that its "late" replication and DNA packaging then proceed according to the lambda mode (Fig. 6). In the case of infection, the circular molecule is formed after circularization of infecting phage DNA via cohesive ends. It is possible that, in the case of lytic development, under certain conditions, this circular monomer could start "late" rolling circle replication, which, in the case of N15, may be DnaG-independent. The primase function could be provided by RepA.

If the lytic cycle is initiated in the N15 lysogen, the circular monomer initially does not exist and should be generated at some step of prophage replication, as shown in Fig. 6. However, prophage replication is DnaG-dependent and thus the lytic replication in this case will be DnaG-dependent also.

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