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Viral terminal protein directs early organization of phage DNA replication at the bacterial nucleoid

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The mechanism leading to protein-primed DNA replication has been studied extensively in vitro. However, little is known about the in vivo organization of the proteins involved in this fundamental process. Here we show that the terminal proteins (TPs) of phages ϕ 29 and PRD1, infecting the distantly related bacteria *Bacillus subtilis* and *Escherichia coli*, respectively, associate with the host bacterial nucleoid independently of other viral-encoded proteins. Analyses of phage ϕ 29 revealed that the TP N-terminal domain (residues 1–73) possesses sequence-independent DNA-binding capacity and is responsible for its nucleoid association. Importantly, we show that in the absence of the TP N-terminal domain the efficiency of ϕ 29 DNA replication is severely affected. Moreover, the TP recruits the phage DNA polymerase to the bacterial nucleoid, and both proteins later are redistributed to enlarged helix-like structures in an MreB cytoskeleton-dependent way. These data disclose a key function for the TP in vivo: organizing the early viral DNA replication machinery at the cell nucleoid.

Bacillus subtilis | phage ϕ 29 | DNA polymerase | DNA-binding | bacterial cytoskeleton

Protein-primed DNA replication is a mechanism used to initiate DNA synthesis in a variety of prokaryotic and eukaryotic organisms (1). Phages such as ϕ 29 (infecting *Bacillus subtilis*) and PRD1 (infecting *Escherichia coli*) (1, 2), animal viruses such as adenoviruses (1, 3), bacterial species from the *Streptomyces* genus (1, 4), and viruses infecting Archaea (5–7) possess replication origins at their linear chromosomes constituted by inverted terminal repetitions with a terminal protein (TP) linked to both 5' genome ends. TPs also have been reported in linear plasmids isolated from bacteria, yeast, fungi, and higher plants (1) and in animal and plant RNA viruses (1).

The development of an in vitro replication system with purified proteins and DNA from the *B. subtilis* phage ϕ 29 laid the foundations for investigating the protein-primed mechanism of DNA replication and makes ϕ 29 a paradigm for studying this process (1, 2). A schematic overview of the in vitro ϕ 29 DNA replication mechanism is shown in Fig. S1. The ϕ 29 genome consists of a 19,285-bp linear dsDNA, with a TP of 31 kDa (the parental TP) covalently linked to each 5' end. DNA replication starts with the recognition of the TP-containing DNA ends by a heterodimer formed by the ϕ 29 DNA polymerase and a free TP molecule (the primer TP) (8). The DNA polymerase then catalyzes the formation of a covalent bond between deoxyAMP and the hydroxyl group Ser²³² of the primer TP. Replication is coupled to strand displacement, and continuous elongation of the DNA polymerase from both DNA ends generates replication intermediates that finally converge in the complete duplication of the parental strands (reviewed in ref. 2). Phage ϕ 29 DNA transcription is divided into early and late stages (9). Fig. S1 shows a genetic and a transcriptional map. Genes 2 and 3, encoding phage DNA polymerase and TP, respectively, are located in the left-side early operon.

Although the protein-priming mechanism of DNA replication has been studied extensively in vitro, the underlying mechanisms

governing the in vivo organization of the proteins involved in this process are poorly understood. Recently, we provided insights into the in vivo organization of proteins involved in ϕ 29 DNA replication by showing that the bacterial actin-like MreB cytoskeleton, playing important roles in several cellular processes (reviewed in 10), is required for efficient DNA replication of the distantly related phages ϕ 29, SPP1, and PRD1 (11). Components of the ϕ 29 DNA replication machinery, such as the DNA polymerase, are redistributed in peripheral helix-like structures in a cytoskeleton-dependent way.

Here, we analyze the subcellular localization of the TP of phage ϕ 29 in live *B. subtilis* cells and show that it associates with the bacterial nucleoid independently of other phage-encoded proteins. The N-terminal domain of the TP directs its nucleoid association and is required for efficient ϕ 29 DNA replication. The TP recruits the phage DNA polymerase to the bacterial nucleoid in early infection, and later both proteins relocate to peripheral helix-like structures in a process dependent on the bacterial cytoskeleton.

Results

Viral TPs Associate with the Bacterial Nucleoid Independently of Other Phage-Encoded Proteins. As a first approach to determine the in vivo organization of viral protein primers in bacteria, we engineered a *B. subtilis* strain containing a xylose-inducible fusion of *yfp* to the ϕ 29 gene 3 cloned at the chromosomal *amyE* locus. Complementation experiments using the ϕ 29 replication-deficient mutant phage *sus3*(91), unable to synthesize TP, showed that the TP fusion protein YFP-TP was functional (Fig. S2A and B).

Fig. 1A shows the localization of YFP-TP in *B. subtilis* non-infected cells. Merged images of both YFP-TP and DAPI fluorescent signals demonstrated that YFP-TP colocalizes with the bacterial nucleoid. Therefore, the nucleoid localization of YFP-TP is independent of other ϕ 29-encoded proteins. Importantly, we found that the TP belonging to the distantly related phage PRD1, fused to CFP, similarly colocalizes with the *E. coli* nucleoid independently of other PRD1 proteins (Fig. 1B), suggesting a conserved functional property of viral TPs in bacteria. Control cells expressing YFP or CFP in *B. subtilis* and *E. coli*, respectively, displayed a fluorescent signal uniformly distributed along the entire length of the bacteria (Fig. 1C). We found that ϕ 29 and PRD1 TPs also are able to associate with nucleoids of *E. coli* and *B. subtilis*, respectively (Fig. 1D).

We next analyzed the localization of the ϕ 29 TP fusion in live *B. subtilis*-infected cells as a function of time. As shown in Fig. 1E, 10 min after infection YFP-TP fluorescent signal occupied

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The authors declare no conflict of interest.

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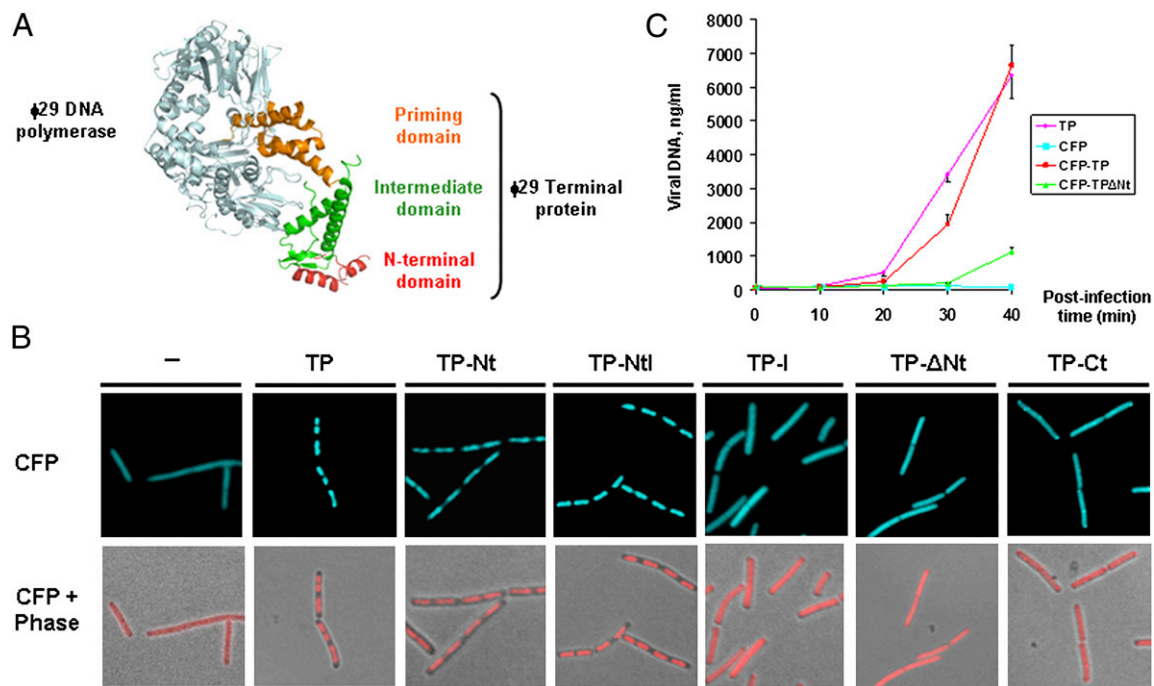


Fig. 2. The TP N-terminal domain localizes at the bacterial nucleoid and is important for efficient ϕ 29 DNA replication. (A) Three-dimensional structure of the DNA polymerase/priming TP heterodimer (12). (B) *B. subtilis* cells expressing CFP (strain DM-024), CFP/TP (strain DM-025), CFP/TP-Nt (strain DM-026), CFP/TP-Ntl (strain DM-027), CFP/TP-I (strain DM-028), CFP/TP- Δ Nt (strain DM-029), and CFP/TP-Ct (strain DM-030) IPTG-inducible fusions were grown to midexponential phase in LB medium at 37 °C. At an OD₆₀₀ of 0.4, the cultures were supplemented with 1 mM IPTG. Samples were harvested and analyzed by fluorescence microscopy 30 min after IPTG addition. For clarity, CFP fluorescent signals are false-colored red in merged images. (C) The amount of intracellular accumulated phage ϕ 29 DNA was quantified by real-time PCR at different times postinfection with a *sus3(91)* mutant phage of the following *B. subtilis* strains: DM-024 (expressing CFP), DM-025 (expressing CFP-TP), DM-029 (expressing CFP-TP Δ Nt), and DM-032 (expressing wild-type TP). Samples were infected at a MOI of 1 and at different times postinfection were processed as described in *Materials and Methods*. The amounts of accumulated phage DNA (nanograms of viral DNA per milliliter of culture) are expressed as a function of time after infection. The experiment was carried out in triplicate to calculate SDs.

protected from DNase I attack with no obvious sequence preference. Together, these results show that a functional property of the TP, binding to dsDNA, resides in the N-terminal domain of the protein.

Efficient ϕ 29 DNA Replication Requires the TP N-Terminal Domain. To assess the importance of the N-terminal domain for the efficiency of *in vivo* ϕ 29 DNA replication, we performed complementation assays using the *sus3(91)* mutant phage and measured by real-time PCR the amount of accumulated viral DNA in *B. subtilis* strains expressing wild-type TP, CFP-TP, CFP-TP Δ Nt, and CFP, respectively. Fig. 2C shows that similar efficient ϕ 29 DNA replication was obtained in *sus3(91)*-infected cells that were complemented *in trans* with either TP or CFP-TP, indicating that the CFP-TP fusion is functional. In contrast, the efficiency of DNA replication in the strain expressing CFP-TP Δ Nt was severely decreased compared with the strain producing CFP-TP. Western blot analysis demonstrated that the amount of proteins expressed *in trans* was similar to that of TP synthesized in a typical ϕ 29 infection cycle (Fig. S3A). Also, analyses of phage DNA accumulation by agarose gel electrophoresis confirmed that the TP N-terminal domain is required for efficient intracellular accumulation of ϕ 29 DNA (Fig. S3B).

ϕ 29 TP Recruits the Phage DNA Polymerase to the Bacterial Nucleoid, and Both Proteins Subsequently Are Redistributed in a Helix-Like Manner. We previously showed that ϕ 29 DNA polymerase localizes uniformly in non-infected cells, and that at middle stages of the infection cycle, it localizes into clear helix-like structures in *B. subtilis* (11). Nevertheless, at 10 min postinfection, the ϕ 29 DNA polymerase fused to YFP (YFP-p2) colocalizes with the bacterial nucleoid (Fig. S4). Because ϕ 29 priming TP and DNA polymerase

interact, forming a heterodimer to initiate phage DNA replication (8), and because the TP is able to localize at the bacterial nucleoid independently of other phage-encoded proteins (Fig. 1A), we analyzed whether the ϕ 29 TP has a role in recruiting the DNA polymerase at the bacterial nucleoid. To do so, we constructed a *B. subtilis* strain able to express simultaneously functional CFP-TP and YFP-p2 fusions (Fig. S2 C–E) from IPTG- and xylose-inducible promoters, respectively. The results show that, in the absence of CFP-TP expression, YFP-p2 distributes uniformly in noninfected cells (Fig. 4A). However, when the growing culture was supplemented with both IPTG and xylose (i.e., when both the CFP-TP and the YFP-p2 fusions were expressed), YFP-p2 relocated to the bacterial chromosome. Thus, the ϕ 29 TP plays a crucial role recruiting the phage DNA polymerase to the bacterial nucleoid.

To unravel further the *in vivo* organization of the ϕ 29 protein priming machinery, we simultaneously examined the localization of the phage TP and DNA polymerase in *B. subtilis*-infected cells. As shown in Fig. 4B, 10 min postinfection, CFP-TP and YFP-p2 colocalized at the bacterial nucleoid. Importantly, 40 min postinfection both CFP-TP and YFP-p2 had relocated and followed similar helical paths. Merging the CFP and YFP signals revealed a substantial colocalization.

Proper Redistribution of ϕ 29 TP to Helix-Like Patterns Requires the MreB Cytoskeleton. We previously demonstrated that the helical localization of the ϕ 29 DNA polymerase at middle stages of infection is lost in *mreB* cytoskeleton mutant strains (11). To investigate whether the helix-like redistribution of ϕ 29 TP also depends on MreB, we examined the subcellular localization of YFP-TP in an *mreB* deletion mutant. As shown in Fig. 4C, and unlike wild-type cells, YFP-TP did not adopt a helical configuration in the MreB cytoskeleton mutant even at late postinfection

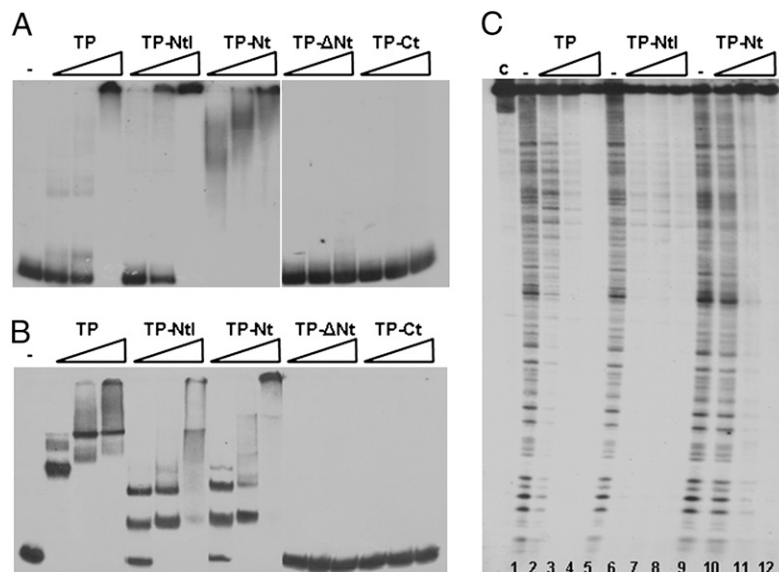


Fig. 3. The N-terminal domain of the TP has sequence-independent dsDNA-binding capacity. Gel mobility shift assays using an end-labeled 297-bp DNA fragment corresponding to the right end of the ϕ 29 genome (A) and an end-labeled 216-bp DNA fragment corresponding to the *B. subtilis yshC* gene (B). The labeled probes were incubated either in the absence (–) or presence of increasing amounts (75, 150, and 300 nM) of the indicated purified protein in a buffer containing 50 mM NaCl. After nondenaturing PAGE, the mobility of the nucleoprotein complexes was detected by autoradiography. (C) DNase I treatment of nucleoprotein complexes formed by ϕ 29 wild-type TP and TP-Ntl or TP-Nt truncated variants. The 297-bp right end of the ϕ 29 DNA fragment was end labeled and used in DNase I footprinting assays in the absence (–) or presence of increasing amounts (0.75, 1.5, and 3 μ M) of the indicated proteins. The bottom of the footprints corresponds to the right end of the ϕ 29 genome. c, control lane with the dsDNA probe not subjected to DNase I treatment and protein preincubation.

times. Instead, the fluorescent signals remained associated to the nucleoid area and were not redistributed in a helix-like manner.

Discussion

Bacterial viruses produce high numbers of progeny within a narrow time window during their lytic cycle. Following infection, phage DNA replication is expected to be organized rapidly, both spatially and temporally, to allow simultaneous amplification of multiple templates in a short period. Based on the experiments described in this paper, a schematic representation of the initial steps of the ϕ 29 infection cycle is shown in Fig. 5.

Our results show that the parental TP (in green and red) covalently linked to each 5' end of the ϕ 29 genome (linear dsDNA, shown as a double helix) associates with the *B. subtilis* nucleoid (gray mass at bottom) at early infection times. This binding occurs independently of priming TP, strongly indicating that, after ϕ 29 TP-DNA injection takes place, the parental TP directs the viral genome to the bacterial nucleoid (Fig. 5A). In this way, the phage ϕ 29 genome gains access to the *B. subtilis* RNA polymerase, which, like the ϕ 29 TP, localizes at the bacterial nucleoid (Fig. S5), initiating transcription of the early genes required for phage DNA replication. Once both ϕ 29 TP and DNA polymerase (shown in cyan) are synthesized, they form a heterodimer that associates with the bacterial nucleoid through the N-terminal DNA-binding domain of the priming TP (shown in red) (Fig. 5B) and recognizes the replication origins at both DNA ends by means of specific interactions with the parental TP (14, 15). It seems likely that the association of the ϕ 29 TP-DNA to a specific bacterial compartment also might help stabilize the interaction with the heterodimeric complex. The TP N-terminal domain has sequence-independent DNA-binding capacity, and no direct interaction between the ϕ 29 TP and nucleoid-associated proteins HBsu, SMC, ScpA, or Noc was detected by yeast two-hybrid assays (Fig. S6). After a transition step, the DNA polymerase dissociates and continues processive elongation (Fig. 5C) until the nascent DNA strand is completed (Fig. 5D). The newly replicated TP-DNA reassociates with the bacterial nucleoid, allowing a new

replication cycle to begin (Fig. 5E). The ϕ 29 strategy at early infection stages is to anchor its genome to the nucleoid, facilitating the access to the RNA polymerase. In agreement with our model, it was shown previously that the ϕ 29 TP-DNA, although not covalently closed, is topologically constrained in vivo (16). Additionally, both novobiocin and nalidixic acid, inhibitors of the DNA gyrase, significantly impair viral DNA replication (16). Hence, the *B. subtilis* DNA gyrase is required for efficient in vivo ϕ 29 DNA replication. This topological constraint might be caused at least in part, as shown here, by the association of the parental TP with the bacterial nucleoid.

After the ϕ 29 DNA replication machinery is associated with the nucleoid, both the TP and the DNA polymerase are reorganized, adopting a peripheral helix-like configuration toward the cell poles. Interestingly, it has been reported recently that, as long as the *B. subtilis* chromosome is being replicated, the two newly synthesized DNA copies are similarly translocated via a peripheral helical structure to the opposite poles (17). Most likely, the TP associates with the newly synthesized bacterial DNA and adopts its morphology during segregation to the future daughter cells. Thus, it is tempting to speculate that ϕ 29 DNA uses the motor-like force that provides chromosome segregation in *B. subtilis* to redistribute its replication machinery at multiple peripheral sites. Conspicuously, convincing evidence has been reported from *E. coli* and *Caulobacter crescentus* that MreB proteins, responsible mainly for the regulation of cell shape, also play a leading role in bacterial chromosome partitioning (18–20). Although the notion that MreB has a direct, mitotic-like role in chromosome segregation in *B. subtilis* remains controversial (21–23), it would explain the observation that essential ϕ 29 DNA replication components (i.e., TP and DNA polymerase) redistribute following helical structures in an MreB cytoskeleton-dependent way. The intrinsic dynamic behavior of the replicating bacterial chromosome during its partitioning would enhance efficiency in organizing ϕ 29 DNA replication, thereby allowing synchronized replication of multiple templates at various peripheral sites.

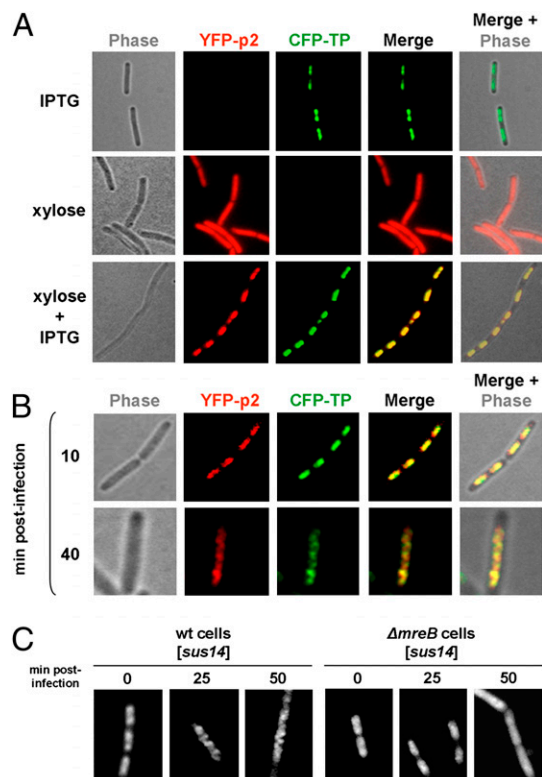


Fig. 4. The ϕ 29 TP recruits the DNA polymerase to the bacterial nucleoid, and both proteins are redistributed in an MreB-dependent way. (A and B) *B. subtilis* strain DM-023, expressing YFP-p2 and CFP-TP in a xylose- and IPTG-dependent way, respectively, was grown in LB medium supplemented with 5 mM $MgSO_4$ and 2% glucose. At an OD_{600} of 0.4, cultures were supplemented with 1 mM IPTG and/or 0.5% xylose, as indicated (A) and simultaneously were infected with a *sus14*(1242) phage at a MOI of 5 (B). Shown are phase-contrast, YFP and CFP fluorescence, and merged images. For clarity, YFP and CFP fluorescent signals are false-colored red and green, respectively. (C) *B. subtilis* strains expressing YFP-TP in a xylose-dependent way under wild-type (DM-021) and $\Delta mreB$ (DM-031) backgrounds were grown in LB medium supplemented with 25 mM $MgSO_4$ and 2% glucose. At an OD_{600} of 0.4, cultures were supplemented with 0.5% xylose and infected with a *sus14*(1242) phage, as indicated. Samples were harvested 25 and 50 min postinfection. In A–C fluorescence signals are shown after deconvolution of an image stack, as a max projection.

The TPs of phage ϕ 29 and of eukaryotic adenovirus have remarkably similar roles in vivo. The adenovirus TP associates with the cell nuclear matrix (24, 25). As in ϕ 29, this association is postulated to direct replication complexes to the appropriate lo-

cation within the cell and, additionally, appears to be essential for optimal transcriptional activity of the adenovirus genome. However, although the adenovirus precursor of terminal protein (pTP), like the ϕ 29 TP, has DNA-binding capacity (26), the binding of pTP to the nuclear matrix is not dependent on cellular chromosomal DNA (25). Instead, it has been proposed that the DNA-binding ability of pTP stabilizes the adenovirus DNA polymerase on partially unwound DNA origins during the initiation of DNA replication (26). We cannot exclude the possibility that the DNA-binding domain of the ϕ 29 priming TP also might play a similar role, locating the DNA polymerase/priming TP heterodimer precisely at the replication origin. Nevertheless, this possibility seems unlikely because hardly any sequence-dependent DNA-binding capacity was observed for the ϕ 29 TP, and because the N-terminal DNA-binding domain of the TP is not essential for amplifying ϕ 29 DNA in vitro (27). Because (i) the ϕ 29 TP localizes at the bacterial nucleoid independently of other phage-encoded proteins; (ii) the ϕ 29 DNA polymerase is recruited by the TP to the bacterial nucleoid; and (iii) efficient in vivo ϕ 29 DNA replication is compromised in the absence of the TP DNA-binding domain, we propose that the ϕ 29 TP plays a significant biological role, attaching the essential ϕ 29 DNA replication machinery at the bacterial nucleoid via a sequence-independent DNA-binding domain. Importantly, in addition to ϕ 29, the TP of the distantly related *E. coli*-infecting phage PRD1 also localizes at the bacterial nucleoid independently of other viral-encoded proteins. Moreover, we show that both PRD1 and ϕ 29 TP also associate with the nucleoid when expressed in bacteria other than their respective hosts. Taken together, these results reveal a functional property of viral TPs in bacteria that might have been conserved during evolution of a prokaryotic viral ancestor. By playing a key role in the organization of phage DNA replication at the bacterial nucleoid, TPs provide another way for viruses to exploit cell resources to optimize the production of numerous progeny.

Materials and Methods

General Methods. Phage ϕ 29 DNA replication is inhibited by the host protein Spo0A (28). Because the *B. subtilis* *spo0A* promoter switch is repressed by an excess of glucose (29), medium was supplemented with 2% glucose. Unless stated otherwise, mid-logarithmically growing *B. subtilis* cultures were infected at a multiplicity of infection (MOI) of 5, and cell samples were harvested and processed at the indicated times after infection. *B. subtilis* cells were transformed by standard procedures, as described (23). Selection for *B. subtilis* transformants was carried out on nutrient agar plates (Oxoid), supplemented with appropriate antibiotics, 0.5% xylose, 1 mM IPTG, or 25 mM $MgSO_4$ when necessary. Details are given in *SI Text*.

Bacterial and Yeast Strains, Phages, and Growth Conditions. Bacterial and yeast strains and phages used are listed in *Tables S1* and *S2*, respectively. The

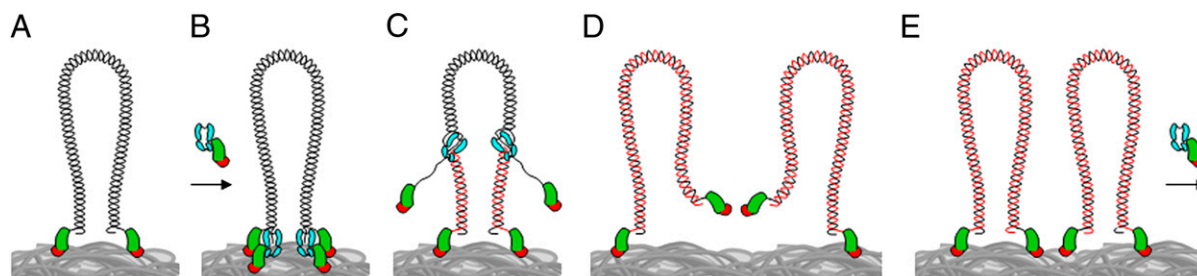


Fig. 5. Model of nucleoid-associated early ϕ 29 DNA replication organized by the TP. (A) A complete ϕ 29 TP-DNA molecule (linear dsDNA shown as a double helix) is shown attached to the bacterial nucleoid surface (gray mass at bottom) by the N-terminal domain (red) of the two parental TPs (red and green). (B) The priming TP interacts with the phage DNA polymerase (cyan), forming a heterodimer that associates with the nucleoid via the TP N-terminal domain and recognizes the origins of replication. (C) After a transition step, the DNA polymerases dissociate and continue processive elongation of the nascent DNA strands (red lines) coupled to strand displacement. (D and E) Once DNA replication is completed, two ϕ 29 TP-DNA molecules are ready for another round of replication. For simplicity, other viral proteins involved in DNA replication are not drawn.

delayed-lysis mutant phage $\phi 29$ *sus14*(1242) contains a mutation in gene 14 that has no effect on phage DNA replication or phage morphogenesis, thus allowing examination of phage protein and DNA localization at late infection times. Details are given in *SI Text*.

DNA Techniques and Plasmid Construction. All DNA manipulations and cloning were carried out by standard methods. Plasmids used are listed in *Table S2*. Details are given in *SI Text*. Oligonucleotides used are listed in *Table S3*.

Protein Purification. Wild-type $\phi 29$ TP was expressed in *E. coli* BL21(DE3) cells harboring gene 3 cloned into plasmid pT7-3 and further purified as described (13). Mutant proteins TP-Nt ($\phi 29$ TP N-terminal domain, spanning residues 1–73), TP-Ntl ($\phi 29$ TP N-terminal and intermediate domains, spanning residues 1–173), TP- Δ Nt ($\phi 29$ TP lacking the N-terminal domain, spanning residues 74–266), and TP-Ct ($\phi 29$ TP priming domain, spanning residues 174–266) were expressed in the *E. coli* strain BL21(DE3). The TP variant TP-Ntl was purified essentially as described for the wild-type $\phi 29$ TP (13). The $\phi 29$ TP variants TP-Nt, TP- Δ Nt, and TP-Ct were purified using Ni²⁺-NTA resin columns.

Gel Mobility Shift and Footprinting Assays. Gel retardation and DNase I footprinting assays were performed essentially as described (30, 31). A 297-bp DNA fragment corresponding to the $\phi 29$ right end was amplified by PCR using genomic $\phi 29$ DNA as template and primers R-25 and R-OUT SUPER. A 216-bp DNA fragment corresponding to the *B. subtilis* *yshC* gene was amplified by PCR using genomic *B. subtilis* DNA as template and primers yshC_U and yshC_L. The PCR products were labeled at one of the 5' ends by treating the appropriate primer with polynucleotide kinase and [γ -³²P]ATP before the amplification reaction.

Immunofluorescence Microscopy. Samples were fixed after the indicated times of infection and processed essentially as described (32). Details are given in *SI Text*.

Epifluorescence Microscopy. For fluorescence microscopy, overnight cultures were diluted in LB medium containing 5 or 25 mM MgSO₄ and grown to early exponential phase at 37 °C. At an OD₆₀₀ = 0.3–0.6, cells were infected at an MOI of 5 with the indicated $\phi 29$ phage (*Table S2*) and supplemented with 0.5% xylose or 1 mM IPTG, as indicated. For live-cell imaging, cells were immobilized on microscope slides covered with a thin film of 1% agarose in water. CFP and YFP fluorescence were detected with a dual CFP/YFP-ET filter (89002; Chroma).

Real-Time PCR. Cells corresponding to 1-mL aliquots of *B. subtilis* cultures, withdrawn at different times after infection, were harvested, processed, and analyzed by real-time PCR essentially as described (33). Details are given in *SI Text*.

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