The generation of concatemeric plasmid DNA in *Bacillus subtilis* as a consequence of bacteriophage SPP1 infection

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**ABSTRACT**

Bacteriophage SPP1 infection of *Bacillus subtilis* cells bearing plasmids induces the synthesis of multigenome-length plasmid molecules. Two independent pathways can account for this synthesis. In one of those, homology to the phage genome is required, whereas in the other such homology is not a prerequisite. In wild type cells both modes overlap. In *dnaB*(Ts), at non permissive temperature, or in *recE polA* strains the main concatemeric plasmid replication mode is the homology-dependent plasmid (hdp) mode. The rate of recombination-dependent concatemeric plasmid DNA synthesis is a consequence of a phage-plasmid interaction which leads to chimeric phage::plasmid DNA. The second mode, which is an homology-independent plasmid (hip) mode seems to be triggered upon the synthesis of a phage encoded product(s) (e.g. inactivation of the exonuclease V enzyme).

**INTRODUCTION**

Plasmid DNA, which is usually smaller in size than the DNA of a bacteriophage genome, can be encapsidated into phage proheads to produce plasmid transducing particles (1). The encapsidation of linear head-to-tail plasmid molecules has been detected in all bacteria tested so far (2–9). The frequency of plasmid containing phage particles is greatly enhanced [102 to 104-fold] when DNA homology between plasmid and phage is provided (6, 7). Beyond this observation, however, the molecular events that trigger the synthesis of packagable concatemeric plasmid DNA are not well understood. Depending on the replication machinery eventually used, two models to explain such phenomena were proposed. In model 1, a phage encoded product is inhibitory for termination of plasmid replication, leading to the formation of plasmid multimers (7). In this case, homology has the effect of introducing, via homologous recombination between phage and plasmid concatemeric molecules, the phage packaging recognition signal (pac site) into the plasmid concatemer. The model implies that synthesis of the plasmid concatemer is homology-independent, but depends on plasmid-initiated replication. In model 2, a single-stranded end of phage DNA invades a homologous region of a double-stranded plasmid molecule. This recombination intermediate is then converted into a replication fork (10, 11). Such a synapsis between a phage replication intermediate and a plasmid molecule would lead to the generation of a phage::plasmid DNA chimera carrying the pac site. In this situation the phage replisome is driving the synthesis of concatemeric plasmid DNA (6, 9).

In certain *Bacillus subtilis* and *Escherichia coli* strains, linear multigenome-length plasmid molecules, as those described above, were observed 'independent of phage infection'. An impairment in the exonuclease V (ExoV) activity [also termed RecBCD in *E. coli* or AddABC in *B. subtilis* (12)] triggers the synthesis of concatemeric plasmid molecules (13–16). This synthesis requires the active RecA/RecE (equivalent proteins from *E. coli* and *B. subtilis* origin, respectively) and DNA polymerase I products (13, 14, 16). This novel form of plasmid DNA synthesis was reported to be dependent on functions involved in DNA recombination but independent of plasmid replication initiation functions or DnaA protein as in the case of oriC plasmids (14, 15). Consistent with this is the fact that such concatemeric plasmid DNA synthesis is partially resistant to chloramphenicol and rifampicin (17). Furthermore, for phage concatemeric DNA synthesis, inactivation of the ExoV enzyme is needed (18, 19, 20).

In this communication, we analyze the replication of plasmids with or without a DNA fragment from SPP1 genome in SPP1 infected *B. subtilis* cells. Our results suggest the existence of two overlapping concatemeric plasmid replication modes following phage infection. Such plasmid replication will be referred to as homology-independent or homology-dependent when the presence of the SPP1 DNA fragment, on the plasmid, has an influence on the rate of concatemeric plasmid replication. Furthermore, we show that phage::plasmid chimeras, carrying the pac recognition signal, can be generated during phage infection.

**MATERIALS AND METHODS**

**Bacterial strains, phages and plasmids**

*B. subtilis* strains YB886 (wild-type) and the isogenic YB1015 (recE4), BG219 (recE6 polA5), BG193 (dnaB37) and BG195 (dnaB19) have been previously reported (21, 22). The phages used were SPP1 wild type and SPP1am109 (23). The *B. subtilis* plasmids used were based either on the pC194

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or pUB110 replicons. Plasmids pC1941, p1958 and pBG59 were previously described (24, 6). Plasmids p1934 and p1936 are pC1941 derivatives in which a pac site containing SPP1 DNA fragment of 840 bp or 197 bp, respectively, was introduced. pBD331 contains a 2.1 kb DNA fragment adjacent to the SPP1 pac region, but lacks the pac site (24).

**Transduction and transformation**

Transducing phage stocks were prepared and transduction was performed as previously described (24). Transformation followed the protocol of Rottländer and Trautner (25). Antibiotic resistant transductants or transformants were selected on TY plates containing 5 μg/ml of chloramphenicol or 35 μg/ml of ampicillin.

**Analysis of plasmid DNA forms**

*B. subtilis* strains YB886 and BG219 bearing plasmids were grown at 37°C to an OD~560~ of 0.45 (about 5.0×10^7 cells/ml) in TY medium supplemented with the appropriate antibiotics, and infected with 5 phage/bacterium. Phage addition marked the time zero of our experiments. At given times, aliquots of equal volume were removed and poured onto frozen TBT (100 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM MgCl) supplemented with sodium azide to 10 mM. In temperature shift experiments, the strain BG193 dnaB(Ts) bearing plasmids was grown at 30°C, to an OD~560~ of 0.45 and transferred to 46°C. 10 min after shift up the cells were infected with 5 phage/bacterium and processed as described above.

Crude cell lysates were obtained and processed as previously described (14). Since the events to be monitored account for less than 1% of total DNA, prior to electrophoresis to avoid DNA saturation in the nylon membrane, to gain sharpness in the transferred bands and linearity in our quantifications, the DNA preparations were treated with a restriction enzyme which digests phage and chromosomal DNA, but not plasmid DNA. Aliquots of crude cell lysates were electrophoresed overnight in 0.8% agarose. The DNA was transferred to a nylon membrane (GeneScreen, NEN) and processed essentially as described by Maniatis et al. (26).

The relative amounts of plasmid DNA forms given in the text refer to the intensities of the plasmids bands present on the autoradiograms. They were quantitatively scanned with a laser densitometer (LKB UltroScan XL). The linearity of the response with respect to DNA concentration was checked using autoradiograms of different exposure times. Quantitative scans were integrated using the LKB GelScan XL software package.

Since plasmids pC1941 and p1958 shared the pC194 replicon (equal copy number ?) and the rate of SPP1 DNA synthesis (correction for the amount of infected cells) in cells carrying either plasmid pC1941 or p1958 does not differ more than 1.4-fold we assumed that normalizing the amount of concetemeric plasmid DNA form to the amount of form I (monomeric and dimeric supercoiled covalently closed plasmid) DNA could represent the in vivo situation.

**Other biochemical techniques**

Plasmid DNA was prepared on preparative and analytical scales as previously described (27). Restriction enzymes and other relevant enzymes were purchased from Boehringer (Mannheim, FRG). All enzymes were used as described by the suppliers. Other standard techniques as DNA hybridization and nick-translation of DNA with [α^32P]dATP (Amersham-Buchler (UK)) were performed as described by Maniatis et al. (26).

Radioactive probes were prepared by nick-translation of the *E. coli* plasmids pBT39 (cat-probe, containing 0.7 kb of pC1941), pBT38 (ble-probe, containing 0.5 kb of pUB110) and pBT43 (pac-probe, containing 0.7 kb of the SPP1 pac region). The probes detect the chloramphenicol (cat) and phleomycin (ble) resistance genes of plasmids pC1941 and pUB110 and the origin of SPP1 packaging (pac), respectively.

**Fig. 1.** Time course of the relative plasmid DNA mass accumulated during SPP1 infection on wild type cells carrying plasmid pC1941 (A) or p1958 (B). Cultures of YB886 cells harboring plasmid were grown at 37°C to OD~560~ = 0.45 and half of the culture was infected with SPP1 (time zero). From the infected (+ SPP1) and non-infected (− SPP1) cultures samples were withdrawn at indicated times. The vertical arrows denote the DNA preparations, obtained 30 min after infection, digested either with EcoRI (A) or Thal (B). The horizontal arrows indicate the position of form I plasmid DNA and the origin of the gel.

**Fig. 2.** Kinetics of accumulation of concetemeric plasmid DNA during SPP1 infection of wild type (A) and dnaB37 (B) strains. The relative amount of concetemeric plasmid DNA is presented as a ratio of concetemeric to form I (monomeric and dimeric) plasmid DNA. The intensities of plasmid bands present on the autoradiograms, after quantitative scan with a laser densitometer, were integrated by the use of the GelScan software package. (●) plasmid pC1941 and (●) plasmid p1958.
RESULTS

Synthesis of multigenome-length plasmid molecules during SPP1 infection

In order to understand how plasmid transducing particles are generated and which role DNA homology plays in this process, we began with the characterization of plasmid DNA forms during phage infection. For this analysis, two plasmids presenting either no apparent (pC1941) or extensive (174 base pairs, p1958) DNA homology to the SPP1 genome were compared. pC1941 is transduced with a frequency of about $10^{-5}$ transductants/surviving cell, whereas p1958 is transduced with a frequency of about $10^{-3}$ transductants/surviving cell (6).

*B. subtilis* YB886 cells carrying plasmids pC1941 or p1958 were grown exponentially at 37°C to about $5.0 \times 10^7$ cells/ml and either infected with SPP1 or allowed to grow further (non-infected cells). At different times, samples from infected and non-infected cultures were collected and total intracellular DNA was purified. The DNA preparations were treated with HpaI or HindIII, in the case of pC1941 or p1958, respectively. These enzymes have recognition sites in the phage and host chromosome, but not in plasmid DNA. The DNA forms were then separated electrophoretically and analyzed by Southern hybridization using the cat plasmid probe.

As shown in Figure 1, after SPP1 infection a slowly moving DNA form, which hybridizes specifically to the cat-probe, is accumulating (Fig. 1A and IB). This plasmid DNA form co-migrates with undigested phage DNA (data not shown). When the total intracellular DNA is also digested with an enzyme that cleaves the plasmid once (EcoRI and HaeIII for pC1941 and p1958 DNAs, respectively) only one signal corresponding to linear unit-length plasmid molecules is observed (Fig. 1A and IB, lane 8).

Thus, the slow migrating DNA form is of plasmid origin and represents a plasmid concatemer which must be organized in a head-to-tail configuration.

Such plasmid concatemeric DNA was neither detected in uninfected cells (Fig. 1A and IB) nor following infection with a pleiotropic suppressor-sensitive SPP1 mutant (amlO9) (data not shown), which synthesizes only a few early phage products under non permissive conditions (see 23). Thus, this *de novo* plasmid concatemeric DNA synthesis is dependent on phage encoded product(s).

The amount of plasmid concatemer detected 30 min after infection is about 15- to 17-fold greater than the amount detected 15 min after infection (Fig. 2A). The synthesis of concatemeric plasmid form has the same kinetics whether the plasmid shares homology or not with the phage DNA (Fig. 2A). Since phage concatemeric DNA synthesis implies inactivation of the ExoV enzyme (18, 19, 20) and, in both *E. coli* and *B. subtilis*, linear head-to-tail plasmid molecules have been detected after ExoV inactivation (13, 14), we interpret that SPP1 promotes the synthesis of such concatemeric plasmid molecules by inactivating the ExoV enzyme.

In spite of the same amount of concatemeric plasmid DNA

<table>
<thead>
<tr>
<th>Donor strain</th>
<th>Relevant genotype</th>
<th>Time of infection after shift (min)</th>
<th>Transductants/surviving cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>YB886</td>
<td>dna+</td>
<td>0</td>
<td>$6.6 \times 10^{-3}$</td>
</tr>
<tr>
<td>BG193</td>
<td>dnaB37</td>
<td>30</td>
<td>$5.1 \times 10^{-3}$</td>
</tr>
<tr>
<td>BG195</td>
<td>dnaB19</td>
<td>0</td>
<td>$4.5 \times 10^{-3}$</td>
</tr>
</tbody>
</table>

(a) The strains containing plasmid p1958 were grown at 30°C. After such growth one half was infected with SPP1 and kept at 46°C till lysis and the other one was incubated for a further 30 min at 46°C prior to infection. The lysates were used to transduce YB 886 cells at 37°C.

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Fig. 3. Effect of the dnaB37 mutation on the accumulation of concatemeric plasmid molecules. Cultures of BG193 carrying plasmid pC1941 (A) or p1958 (B) were grown at 30°C to OD$_{560}$ = 0.45 and transferred to 46°C. 10 min after temperature shift up, the cultures were infected with SPP1 (time zero). Experimental conditions are like in Fig. 1.

Fig. 4. Effect of the recE6 polA5 mutations on the accumulation of concatemeric plasmid molecules. Cultures of BG219 carrying plasmid pUB110 (A) or pBG59 (B) were grown at 37°C to OD$_{560}$ = 0.45 and infected with SPP1 (time zero). Experimental conditions were those of Fig. 1.
DNA synthesis in the absence or presence of homology, the striking difference between the transduction frequencies in the two cases remain. p1958 (6.4 x 10⁻³ transductants/surviving cell) is transduced with a 230-fold higher efficiency than pC1941 (2.7 x 10⁻⁵ transductants/surviving cell) (6).

Homology-dependent plasmid replication during SPP1 infection

It is known that phage SPP1 and pC1941 or p1958 plasmid DNA replication are independent of the B. subtilis DnaB protein which is a component of the prereplisome (23, 16). However, the host DnaB protein is required for the initiation of chromosomal DNA replication (28) as well as for multigenome-length plasmid DNA replication (17, Viret and Stiege, personal communication). Furthermore, in the latter DNA synthesis the recE mutation (equivalent to recA of E. coli) and polA gene products are involved (13–16). In order to test whether the synthesis of multigenome-length plasmid molecules following SPP1 infection is only due to an indirect effect [replication as a consequence of inactivation of the ExoV enzyme (13, 14)] or due to some other mode of plasmid replication (see Introduction, model 2), the rate of concatemeric plasmid DNA synthesis following SPP1 infection was measured in different genetic backgrounds.

B. subtilis dnaB37(Ts) cells bearing plasmid pC1941 or p1958 were shifted from 30°C to 46°C. 10 min after the shift to non-permissive temperature, cultures were infected with bacteriophage SPP1. At given times samples were collected, total intracellular DNA was purified and processed as described above.

After the temperature shift, chromosomal replication was inhibited as expected for a dnaB mutant strain (data not shown). The amount of slowly moving pC1941 DNA detected in the dnaB(Ts) strain (Fig. 3A) is markedly reduced and follows different kinetics (Fig. 2) when compared to the amount detected in the wild type strain (Fig. 1A). In contrast, slowly moving p1958 DNA molecules are detected about 10 min after infection (Fig. 3B) and its kinetics of synthesis is similar in dnaB and wild type cells (Fig. 2). Thus, a) thermal inactivation of the DnaB product affects homology-independent concatemeric plasmid DNA synthesis (hip), and ii) homology between the phage and the plasmid DNA compensate for the absence of the DnaB product. Hence, we termed this mechanism homology-dependent plasmid (hdp) replication mode.

The amount of concatemeric plasmid DNA 20 min after infection is about 28-fold higher for p1958, than in the case of pC1941 (Fig 2B). However, at later times pC1941 concatemeric DNA accumulates (Fig. 2B). This limited accumulation of pC1941 concatemeric DNA might be due to leakiness of the dnaB37(Ts) mutation. Alternatively, the slower-moving plasmid DNA form in cells bearing plasmid pC1941 could be synthesized at a lower efficiency by a hdp mechanism due to a short stretch of natural homology between the phage and the plasmid (6). 30 min after infection the amount of head-to-tail concatemeric plasmid DNA is still more than 8-fold higher in p1958 than in pC1941.

p1958 transduction frequency of phage stock lysates amplified at 46°C in the wild type, dnaB37(Ts) or dnaB19(Ts) strains bearing plasmid does not differ more than two fold (see Table 1). To confirm whether homology plays any role in the synthesis of concatemeric plasmid DNA, we have used the recE6 polA5 strain. Since, the recE6 polA5 strain by construction is resistant to chloramphenicol (recE6 = recE::cat insertional inactivation) (22), the neomycin-resistant plasmids pUB110 (no homology) and pBG59 (with homology) were used. Transduction in a wild type background of such plasmid DNA by phage SPP1 is identical to the one observed with the pC1941 derived replicons (6). Here, BG219 (recE6 polA5) cells bearing plasmid pUB110 or pBG59...
The kinetic of synthesis of pBG59 concatemeric DNA amount synthesized in the presence of homology (Fig. 4). The delay and its amount markedly reduced when compared to the recE6 polA5 strain does not differ more than two-fold when compared with the recE+ cells (YB886) (data not shown).

From those data we can conclude that the synthesis of concatemeric phage, hdp and hip DNA replication are closely linked in time, but hip replication could become unlinked either by thermal inactivation of the DnaB product or by mutations in the recE and polA genes. DNA homology between the plasmid and the phage is necessary for hdp replication.

**Phage::plasmid chimeras are generated during the synthesis of concatemeric plasmid DNA**

All virus packaging apparatus recognize the nucleic acid to be packaged by virtue of a specific base sequence in such nucleic acid [packaging recognition signal (pac) site] (29)]. In SPP1 DNA homology between the plasmid and phage genomes, irrespective of whether this includes the pac signal on the plasmid, greatly increases the amount of concatemeric plasmid DNA encapsidated into phage proheads (24). How does the phage packaging machinery recognize the plasmid concatemer as a substrate for encapsidation? A simple assumption is that, in a ‘homology-dependent fashion’, a concatemeric plasmid::phage chimeric molecule is generated. Such hybrid molecule would contain the phage pac site. In the case of SPP1, packaging of such chimera would proceed sequentially and unidirectionally from the pac site (35, 31). To test the above possibility, stock lysates of wild type cells bearing pC1941-derived plasmids containing SPP1 DNA fragments from regions near pac (pac-free pBD331) and pac-containing (p1934 and p1936) plasmids were obtained. Total packaged DNA was purified, digested with AliII (cleaves the plasmids once, but does not cleave phage DNA), and analyzed by Southern blot using as probe nick-translated pC1941 DNA. In addition to a major DNA band, which corresponds to unit-length plasmid molecules, a discrete minor band was detected (Fig. 5A). The length of the minor band is 3.9 kb, 2.7 kb or 2.0 kb for plasmids pBD331, p1934 or p1936, respectively. The size of such fragments suggest that in all cases the phage packaging signal is utilized (see Fig. 5B). Since pBD331 does not have a pac site, we infer that the plasmid gained the pac site from the phage. A similar observation was reported for the Staphylococcus aureus phage phi1-P181 system (7). These results reflect that, even in the absence of a pac site in the region of plasmid::phage homology, concatemeric plasmid::phage chimeras carrying pac must be generated during SPP1 infection.

Further evidence was obtained by analyzing total DNA (without pre-digestion, see Materials and Methods) from wild type or dnaB37 infected cells bearing plasmid p1958 (20 and 30 min after infection). Such DNA was digested with AliII, AliII-EcoRI, PvuII-EcoRI or HindIII and analyzed by Southern blot using either cat (plasmid) or pac (phage) probes. No AliII, but multiple recognition sites for EcoRI and HindIII are present in the SPP1 DNA; whereas p1958 is cut only once with AliII and PvuII and does not have any recognition site for EcoRI and HindIII. Since cleavage at the SPP1 pac site occurs about 12 min after infection (our unpublished results), the experiments were designed with the following criteria in mind: i) the stretch of homology between the plasmid and the phage is 2.2 kb away from the phage pac site (30, Fig. 6C), and ii) the restriction enzymes and specific radiolabeled DNA probes chosen would allow us to analyze whether the putative phage::plasmid chimeras shown in Fig. 6C

![Fig. 6. Southern analysis of SPP1 infected B. subtilis cells carrying plasmid p1958.](image-url)

(A) Total DNA obtained 30 min after infection of YB886 cells was digested with EcoRI and AliII (1) or EcoRI and PvuII (2), and blot hybridized with the cat probe. The arrows indicate the position of minor bands (1.9 and 3.0 kb) and the asterisk indicates a band of 1.7 kb. (B) Total DNA obtained 20 min (1, 3 and 4) and 30 min (2, 5 and 6) after infection of strains YB886 (1, 2, 3, and 5) or hBD93 (4 and 6) were digested with AliII (1 and 2) or HindIII (3, 4, 5 and 6) and analyzed using the pac probe. Arrows denote the minor band of 3.8 kb and the position of the HindIII resistant DNA molecules. The molecular weight marker (MWM) is SPP1 EcoRI digested. (C) Structure of phage::plasmid chimeras detected in SPP1 infected cells. Open bars represent phage DNA, closed bars denote concatemeric plasmid DNA, the striped box shows the homologous region between the phage and the plasmid. The arrow and the closed square indicate the phage pac site. The wavy lines under the phage and plasmid molecules denote their respective probes (pac and cat probes). The lengths of the DNA segments (in kb) are indicated. Abbreviations: A, AliII; E, EcoRI; H, HindIII; He, HindIII; P, PvuII.
are generated. Two possible crossover products would result, one with phage sequences to the right of the homologous region (I) and the other with phage sequences to the left (II). It should be noted that, only chimera I carrying a pac site would be the substrate for plasmid packaging.

When total DNA from wild type infected cells bearing plasmid p1958 was digested with AfII-EcoRI or PvuII-EcoRI and analyzed with the cat probe (Fig. 6A), an expected signal with the plasmid linear form (2.5 Kb) and a weak signal to a 1.9 Kb fragment (lane 1, AfII-EcoRI) or 3.0 Kb fragment (lane 2, PvuII-EcoRI) were detected (see chimera II). At present, we are unable to explain the signal of a 1.7 Kb fragment which appears when total DNA is digested with PvuII-EcoRI (Fig. 6A, lane 2).

When total DNA was digested with AfIII and analyzed using the pac probe, an expected signal with undigested SPP1 DNA and a weak signal corresponding to a 3.8 Kb fragment were detected (Fig. 6B, lane 1 and 2). This DNA fragment, which is detected as early as 20 min after infection of wild type (Fig. 6B, lane 1) or dnaB(Ts) cells (data not shown), might correspond to the AfIII-pac generated fragment (see chimera I). That this 3.8 kb pac-ended DNA fragment, after AfIII digestion, is of p1958 origin was confirmed by hybridization with the plasmid probe (data not shown). Digestion of total DNA of infected cells bearing plasmid p1958 with HindIII gave rise to the expected SPP1 HindIII fragments 1 and 6 (30) plus a band that co-migrates with the pac site (35, 31). The substrate, consisting of tandemly repeated units arranged in a head-to-tail configuration, is packaged by an unidirectional and progressive ‘headful’ mode (29, 35, 31). Since the homology between phage and plasmid enhances the plasmid transduction frequency (2, 6, 7, 9) and is a prerequisite for the integration of plasmid DNA, the DNA synthesis of pacDNA is independent of plasmid encoded transduction frequency (2, 6, 7, 9, 15, 34). In contrast, plasmid replication was required for the synthesis of concatemeric plasmid molecules in the S. aureus phage phi11-pT181 system (7).

The DNA to be packaged into SPP1 capsids is recognized and cleaved by phage-encoded proteins at a target sequence termed pac (35, 31). The substrate, consisting of tandemly repeated units arranged in a head-to-tail configuration, is packaged by an unidirectional and progressive ‘headful’ mode (29, 35, 31). Since the homology between phage and plasmid enhances the plasmid transduction frequency (2, 6, 7, 9) and is a prerequisite for the integration of plasmid DNA, the DNA synthesis of pacDNA is independent of plasmid encoded transduction frequency (2, 6, 7, 9, 15, 34). In contrast, plasmid replication was required for the synthesis of concatemeric plasmid molecules in the S. aureus phage phi11-pT181 system (7).

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