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Recombination-Dependent Concatemeric Plasmid Replication

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INTRODUCTION

DNA replication and recombination occur simultaneously in the cell and are tightly interrelated. This functional relationship could arise because (i) replication and recombination functions may be expressed simultaneously (e.g., *dnaN* and *recF* gene expression in *Escherichia coli* cells [5, 52]), (ii) some products may be required for both mechanisms (the RecA protein is involved in recombination [54] and in stable DNA replication [32, 43]), or (iii) DNA recombination intermediates may be used as substrates by the replication machinery (T4 secondary replication mode [47]) or vice versa (postreplication repair [55]).

The present review summarizes our knowledge of the mechanisms underlying a novel mode of plasmid replication and its relationship with recombination. This mode, which results in the accumulation of linear concatemers, appears to escape plasmid regulatory functions involved in the control of copy number but relies on host recombination functions. The latter property, shared in common with late phage replication, makes plasmid concatemeric replication an attractive system for the study of DNA replication and recombination in prokaryotes. We hope that this discussion will also help unravel the mechanisms underlying processes such as phage-mediated plasmid transduction, which is an important means of genetic transfer in bacteria, as well as the implication of recombination-initiated replication in the repair of DNA double-strand breaks.

DNA REPLICATION MODES

Replication Mechanisms Unlinked to Recombination

Two modes of replication, which are thought to be unlinked to recombination, have been reported for circular DNA molecules in bacteria. These modes were conventionally named theta and sigma, according to the characteristic structures of replication intermediates.

The theta mode (Fig. 1, a1 to a4), which is used by most known plasmids as well as by the bacterial chromosome, is characterized by the fact that the sites for priming of leading- and lagging-strand syntheses are located close to one another within the origin of replication. From the origin on, replication leads either unidirectionally or bidirectionally to dimers of the replicon, which are finally resolved into monomeric rings. During the whole process, except that resolution step (Fig. 1, the step from a3 to a4), both DNA strands remain covalently closed. Until recently, the theta mode was the only one reported for bacterial plasmids (for a review, see reference 58).

The sigma mode of replication (Fig. 1, b1 and b5) is known to occur during the life cycle of the single-stranded DNA (ssDNA) bacteriophages of members of the family *Enterobacteriaceae* (7, 34) and was more recently proposed for a collection of small plasmids present in gram-positive bacteria (29, 49, 66) and archaebacteria (61). In this case, priming events for leading- and lagging-strand syntheses are temporally and spatially unlinked, occurring at two distinct origins. Unlike theta replication, sigma replication is characterized by a break-dependent initiation mechanism; indeed, one of the DNA strands is nicked by the action of a plasmid-encoded sequence-specific nuclease, the Rep protein (Fig. 1, the step from b1 to b2). The free 3′ OH end generated is then utilized as a primer for leading-strand DNA synthesis (the step from b3 to b4). Usually after one full round of replication the Rep protein terminates the strand displacement at its specific recognition sequence, generating two unit-length circular products, a single-stranded and a double-stranded one (Fig. 1, the step from b4 to b5) (29, 49). Depending on the plasmid system, priming of lagging-strand synthesis occurs before (Fig. 1, b4) or after termination of leading-strand synthesis. This replication mode generates mainly unit-length circular plasmid molecules (14).

Although the term "rolling-circle" is often used in place of "sigma" to designate the mode of replication of ssDNA phages and of small high-copy-number plasmids found in gram-positive bacteria, we propose to keep the former designation for systems which generate long linear concatemers via recombination-dependent replication (25).

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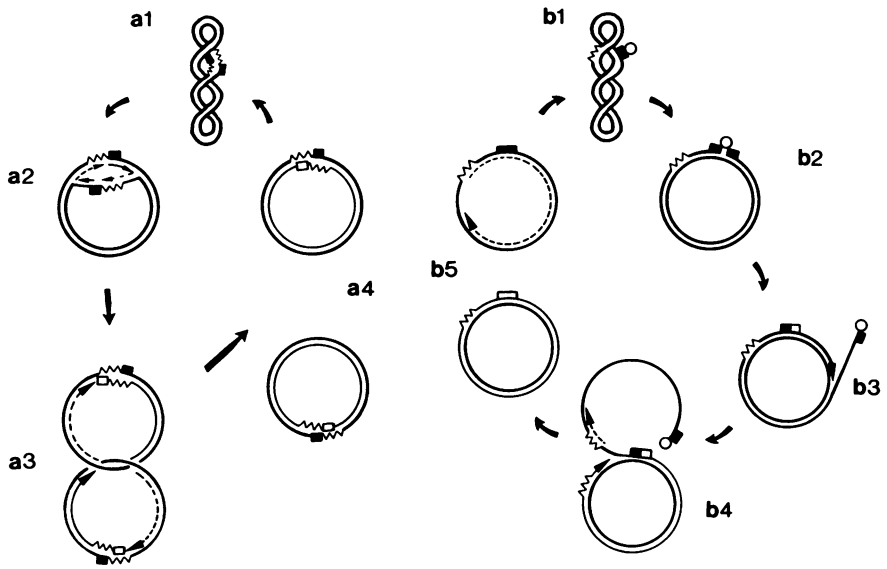

FIG. 1. Theta (a1 to a4) and sigma (b1 to b5) DNA replication modes. For the sake of simplicity, only the elements essential for describing the specific features of each mode have been represented (e.g., the proteins involved in the unwinding and replication of DNA strands are not depicted). a1 to a4 illustrate the situation of a bidirectionally replicating molecule. For a more detailed description, refer to the text. Symbols: ▬ and ▭, leading-strand replication origin of parental and daughter DNA strand(s), respectively; ∿, lagging-strand origin(s); ○, plasmid-encoding Rep protein. Parental DNA strands are represented by heavy lines. Continuous and broken lines represent leading and lagging strands, respectively, with the direction of polymerization shown by an arrow.

Recombination-Dependent DNA Replication

Two mechanisms of DNA synthesis in which replication and recombination are tightly interrelated were first proposed as models for the production of concatemeric molecules during late bacteriophage replication: (i) the rolling-circle mechanism (21, 25) and (ii) a replication mode involving highly branched molecules (47). Recently, the rolling-circle model of replication was identified in plasmids of both gram-negative (18) and gram-positive (69) bacteria. This novel mode of plasmid replication leads to the accumulation of high-molecular-weight (*hmw*) linear head-to-tail plasmid molecules and was observed in certain genetic backgrounds and/or for genetically engineered plasmids (37–39). A similar mechanism might also account for the production of plasmid concatemers in phage-infected cells, a prerequisite for phage-mediated plasmid transduction by various phages (2, 10, 35, 50).

The involvement of recombination proteins in the process of DNA replication has been reported for the bacterial genome as well. In RNase H-defective (*rnh*) mutants of *E. coli*, an alternative mode of chromosomal DNA replication, the so-called constitutive stable DNA replication, was observed (32). Such a replication mode can also be elicited in *rhn*$^+$ cells as a component of the SOS response through the interference with either DNA replication or *rhn* gene expression (inducible stable DNA replication) (33, 53). In both cases, DNA replication is independent of the DnaA function involved in the specific initiation at *oriC*. On the other hand, stable DNA replication relies on the RecA function at some initiation stage(s) (32). Recently, the derepression of the *recA* gene and an increase in the level of RecA protein were shown to be necessary and sufficient for the induction of stable DNA replication via a mechanism involving the RecBCD enzyme and most probably the RecA recombinase activity (43). It is thought that, once initiated, induced stable DNA replication follows the theta mode.

MOLECULAR NATURE OF LINEAR PLASMID CONCATEMERS

In both *E. coli* and *Bacillus subtilis*, the deficiency in exodeoxyribonuclease V (ExoV; also termed RecBCD and AddABC in *E. coli* and *B. subtilis*, respectively) activity correlates with the accumulation of *hmw* linear head-to-tail plasmid concatemers (18, 69). Such a plasmid DNA form has been reported for various unrelated *E. coli* plasmids that use the theta mode of replication, including ColE1-type, mini-λ, mini-F replicons, and minichromosomes (*oriC* plasmids) (18, 37, 59), as well as for *B. subtilis* replicons that replicate via either the sigma or the theta mode (63a, 69, 70). In addition, *hmw* material was observed in wild-type *B. subtilis* cells for genetically engineered plasmids replicating via the sigma mode (28, 36–38, 69, 70). The accumulation of *hmw* DNA even in the ExoV-proficient cells could be explained either by the fact that ExoV binds poorly to long ssDNA tails (see below) or by a titration of the enzyme.

The linear double-stranded nature of the major part of *hmw* plasmid DNA was demonstrated by its sensitivity to exonucleases of known specificity (ExoV, exonuclease III [ExoIII], and the product of the λ *exo* gene) (18, 37, 39, 69). Electron-microscopic examination of the purified *hmw* material from *E. coli sbcB sbcC* mutants expressing the lambda *gam*$^+$ and *red*$^+$ functions (see below) revealed the presence of single-branched circles (σ structures) and linear double-stranded DNA (dsDNA) multimeric plasmid molecules with ssDNA tails (60).

Examination of the purified *hmw* material from a sigma replicating plasmid in wild-type *B. subtilis* cells revealed the presence of three types of linear plasmid DNA molecules: ssDNA, dsDNA, and dsDNA with an ssDNA tail. These different types of molecules, with lengths up to 100 kb, were observed at a similar frequency. Heteroduplex analysis of the dsDNA molecules with an ssDNA tail, using single-stranded specific circular probes, showed that the ssDNA

corresponds to either strand (39, 69). This implies that unlike the replication forks initiated at the leading-strand origin for sigma plasmid replication, the forks giving rise to the concatemers progress either clockwise or counterclockwise on the plasmid template, as reported for λ rolling-circle replication (8, 65). Mapping of the *hmw* molecule ends revealed that, for molecules running with the bulk of purified *hmw* DNA, the position of the single-stranded tail is random, whereas the one of the opposite, double-stranded, end (the putative replication start point [see below]) presents a bias (39). Indeed, independently of which strand is present in the tail, 25% of the double-stranded ends map within either of the plasmid origin regions for sigma replication (39). Since (i) the origin would be in an active orientation in only half of these molecules (1, 70) and (ii) plasmid rolling-circle replication is independent of sigma replication initiation-specific functions (69) (see below), we assumed that this preference might be due to some particular DNA structure typical of origin regions (hot spot) that makes them more susceptible to ssDNA or dsDNA breaks. A similar preference toward the origin region was observed under certain conditions for λ rolling-circle replication (see below).

An ssDNA nick or a dsDNA break in a circular plasmid molecule is supposed to switch on the production of *hmw* concatemers. The concatemers consist of ssDNA, dsDNA, and dsDNA molecules with an ssDNA tail of either strand polarity. The mechanism involved and the topology of DNA molecules observed suggest certain similarities with the late stages of phage DNA replication.

SYNTHESIS OF CONCATEMERIC DNA MOLECULES IN THE BACTERIOPHAGE LIFE CYCLE

The recombination-dependent secondary mode of T4 DNA replication and λ late DNA replication are both responsible for the production of linear concatemeric DNA molecules. We hypothesize that owing to their partially common requirements (see below), the generation of *hmw* plasmid concatemers makes use of mechanisms similar to late phage DNA replication. Therefore, a brief description of the latter systems will provide a frame for understanding the corresponding plasmid mechanism. For a more complete picture, the reader is referred to reviews by Skalka (62), Furth and Wickner (24), and Mosig (47).

A general requirement for the replication of linear molecules in bacteria is the protection of dsDNA ends from exonucleolytic degradation (22, 30, 56). Such protection can be mediated either through the attachment of a protein, such as gp2 of phage T4, to the ends of the bacteriophage genome (26) or via interference with the major host exonuclease (ExoV enzyme). The latter possibility is illustrated for bacteriophage λ: the phage-encoded Gam protein (68) inhibits ExoV activity, which was proposed to degrade some early intermediate structures of λ rolling-circle replication (27). The inhibition apparently results from the binding of the Gam protein to the enzyme (67).

After infection of cells with λ particles or after induction of lysogenic cells, the circular phage DNA replicates via a theta mode (early DNA replication). This replication depends on both λ-encoded replication proteins and several host functions known to be involved in host chromosomal replication (24). The production of linear concatemeric DNA molecules through the rolling-circle mode of replication is triggered roughly at the same time as theta replication and goes on for the entire lytic cycle. Theta replication, however, stops about 16 min after infection (9, 15, 16, 21, 22, 25). Unlike theta replication, the rolling-circle mode relies on recombination functions that are both host and phage encoded.

The λ Gam protein is thought to favour rolling-circle replication by inhibiting ExoV. The mechanism by which an active ExoV prevents the appearance of the λ rolling circle, however, is by degrading DNA structures with double-stranded ends (22, 62). Therefore, the initiation of rolling-circle replication does not appear to be controlled by some phage-encoded regulatory function(s). The free ends of rolling-circle tails are located at many sites on the λ genome (65). Under limiting conditions for initiation of λ theta replication, however, about one-half of the concatemers that were produced via rolling-circle replication were shown to originate at or near the λ origin for theta replication (8).

Recombination functions are also important for phage T4 DNA replication. Mosig (47) proposed a model to explain how recombination events may generate replication forks via the so-called secondary initiation process. According to this model, the 3′-OH end of a T4 DNA molecule that is left single stranded after complete replication of the linear phage genome may invade a double-stranded homologous region present on a second molecule. This recombination intermediate would be a prerequisite for secondary initiation. The invading 3′-OH end acts as a primer for leading-strand synthesis on the invaded molecule. Several T4-encoded functions are involved in the mechanism of secondary initiation, including UvsX (the RecA analog of T4), which would mediate the initial synapsis event, the gp46/47 exonuclease, the gp39/52/60 (type II topoisomerase), and two proteins, gp*uvs*Y and gp59, whose roles are unknown (47).

The DNA to be packaged into a phage capsid consists of tandemly repeated units of the phage genome in a head-to-tail configuration. The presently accepted picture suggests that DNA replication producing such a substrate could proceed via (i) the extension of a 3′-OH end on a randomly nicked circular DNA molecule (rolling circle [62]) or (ii) a priming step involving an invading 3′-OH end on either a convalently closed template (bubble migration [23]) or a double-stranded linear template (late T4 replication [47]) (see below). Any one of these alternative pathways could generate a replication fork and lead to a concatemeric phage DNA molecule, provided the nascent double-stranded end was protected from degradation by the ExoV complex.

FUNCTIONS REQUIRED FOR PLASMID CONCATEMERIC DNA SYNTHESIS

As already pointed out, concatemeric plasmid DNA synthesis appears to share some common properties with phage late replication. Genetic requirements for the accumulation of plasmid concatemeric molecules in *E. coli* and *B. subtilis* are summarized in Table 1. Requirements are listed separately for DNA recombination functions and DNA replication functions.

As far as recombination functions are concerned, the first requirement identified was the inactivation of ExoV (18, 48, 69). In the *E. coli* system, simultaneous inactivation of ExoV and either the inhibition of DNA exonuclease I (ExoI) (*recBC sbcB* double mutants) or the derepression of DNA exonuclease VIII (ExoVIII) activity (*recBC sbcA* double mutants) showed synergistic effects, diverting most of plasmid replication activity from production of circular monomers to synthesis of linear multimers (18). The inactivation of ExoIII (the product of the *xthA* gene) is an alternative way of producing *hmw* DNA in a *recBC* background (48). In agreement with these observations, the accumulation of

TABLE 1. Genetic requirements for plasmid concatemeric replication

Function	Requirement for recombination or replication functions:			
	E. coli		*B. subtilis*	
	Gene	Required	Gene	Required
Recombination				
ExoV	*recBCD*	+	*addABC*	+/−[a]
RecA	*recA*	+	*recA*[b]	+
RecF	*recF*	+	*recF*	−
PolI	*polA*	ND[c]	*polA*	+
Replication				
Initiator product	*dnaA*[d]	−	*rep*(Ts)[e]	−
DNA PolIII[f] holoenzyme	*dnaENQ(ZX)X*[g]	+	*dnaFN(ZX)X*[g]	+
"Primosome components"	*dnaBC, dnaG*	+	*dnaB*,[h] *dnaG*	+

[a] Dispensable for some recombinant plasmids (see text).
[b] The *B. subtilis recA* gene was formerly termed *recE*.
[c] ND, not determined.
[d] For *oriC* plasmids.
[e] Temperature-sensitive plasmid-encoded replication protein (Rep).
[f] Only five (*E. coli*) or four (*B. subtilis*) of the DNA PolIII holoenzyme subunits were characterized on a genetic basis.
[g] The *dnaX* gene was formerly termed *dnaZ* and *dnaX*.
[h] Biochemically uncharacterized.

plasmid concatemeric molecules could also be switched on in an *sbcB* mutant strain, via induction of the λ Gam function, indicating that conditional expression of the latter function is equivalent to the *recBCD* deficiency in promoting *hmw* plasmid DNA synthesis (51, 59). Furthermore, the expression of the *red*$^+$ and *gam*$^+$ genes of bacteriophage λ in wild-type *E. coli* cells leads to the accumulation of *hmw* plasmid DNA (60).

In the *E. coli recBC* background, inactivation of ExoI and activation of ExoVIII are known to open the RecF and RecE pathways of recombination, respectively (see reference 17 for a review). The importance of the recombination state of the host for the generation of *hmw* plasmid molecules is made even more evident by the observations of Silberstein and Cohen (59), later confirmed and extended by Kusano et al. (37). These authors showed that mutations in most of the genes involved in the RecF recombination pathway, including *recA*, *recF*, *recJ*, *recO*, and *recQ*, affected the synthesis of linear plasmid concatemers. Such an effect was observed either in the *recBC sbcB* background or under conditions of *gam* expression in the *sbcB* background. On the other hand, mutations in both *recN* and *ruv*, which also contribute to the RecF pathway, had no such effect.

As quoted above, *hmw* plasmid DNA, sometimes in very large amounts, was also observed in *B. subtilis rec*$^+$ cells bearing recombinant plasmids replicating via the sigma mode (28, 38, 39, 69, 70). The best-characterized system showed a direct correlation between the size of the DNA insert and the amount of concatemeric plasmid DNA produced, with a corresponding decrease in the number of segregable supercoiled plasmid molecules (38). Thus, as observed in *E. coli* (48), the presence of large amounts of linear concatemers may exert a negative effect on normal plasmid replication. Such an effect is generally accompanied by a reduced growth capability and poor viability of the host cells (4a, 37–39). Although the molecular basis of such an interference with plasmid and cell physiology is unknown, we speculate that it may result from the redirection of intracellular pools of enzymes involved in DNA metabolism toward *hmw* DNA synthesis.

In both *E. coli* and *B. subtilis*, a strong dependence of *hmw* plasmid DNA synthesis could be demonstrated for the corresponding enzyme able to catalyze homologous DNA pairing, namely RecA (18, 69). The more than 100-fold reduction in the amount of *hmw* DNA in *recA* strains, which is also observed upon inactivation of the ExoV enzyme, argues against simply an elevated degradation rate of *hmw* DNA in *recA* strains (37, 38). In *B. subtilis*, DNA polymerase I (PolI) also appeared to be required (69, 70). As shown in Table 1, the generation of *hmw* plasmid DNA in the *B. subtilis addA* strain was unaffected by the presence of an additional *recF* mutation, whereas it was prevented in the *addA polA* and *addA recA* double-mutant strains (69, 70; our unpublished results). Since an *addA recF* double mutant (analogous to *E. coli recB recF*) is greatly impaired in both chromosomal and plasmid transformations, as well as in its DNA repair capability (4), these results indicate that, in contrast to the *E. coli* system, the RecF product is not essential for concatemeric plasmid replication. It is, however, possible that another unidentified function can play the same role. A requirement for PolI has not been reported so far for *E. coli*.

Plasmid concatemeric DNA synthesis is independent of rate-limiting functions required for initiation of DNA replication. This could be demonstrated both for a theta-replicating *E. coli oriC* minichromosome, for which the DnaA protein acts as the initiator protein, and for a sigma-replicating plasmid in *B. subtilis* (39, 59, 69). Furthermore, unlike sigma plasmid replication, *hmw* DNA synthesis is partially insensitive to rifampin and chloramphenicol (3, 39). Accordingly, in both organisms the mechanism of replication leading to linear plasmid concatemers appears to be out of the control of the plasmid copy number regulatory functions (59, 69). Conversely, the presence of *hmw* DNA may in some instances exert a negative effect on circular plasmid replication, resulting in segregational instability (37, 38).

As expected, concatemeric plasmid DNA synthesis requires functions involved at later and more general steps of the replication process such as primosome components and DNA polymerase III (PolIII) (Table 1). The *B. subtilis* DnaB protein, which is thought to be a component of the replisome (3, 70), is known to be required for the initiation of chromo-

somal DNA replication (71). In addition, the DnaB product was shown to be involved in *hmw* plasmid DNA synthesis, whereas it is not required for sigma replication of naturally occurring plasmids (3, 39, 70).

Concatemeric plasmid DNA synthesis and late phage DNA replication modes share many common requirements for host DNA recombination and replication functions. The ensuing overall picture suggests that similar mechanisms are operative in both systems. Like late phage DNA replication, the accumulation of *hmw* plasmid concatemers reduces normal plasmid replication and even interfere with cell physiology.

RECOMBINATION-DEPENDENT REPLICATION OF PLASMIDS DURING PHAGE INFECTION

Plasmids which are usually smaller than a bacteriophage genome can be encapsidated into phage proheads to produce plasmid-transducing particles (42). The encapsidation of linear plasmid concatemers has been detected in all bacterium-phage systems tested so far (2, 13, 19, 35, 40, 41, 50, 57, 64). Plasmid transduction frequencies are generally low, about 10^{-5} to 10^{-7} per active phage particle. However, when homology is provided between the phage genome and the plasmid, the transduction frequency increases up to 10^5-fold (transduction facilitation effect) (19, 50, 57). With *B. subtilis* phage SPP1, homology of as few as 47 bp provided a maximal facilitation effect (2).

The mechanism leading to the production of packageable plasmid DNA concatemers after phage infection has not yet been totally elucidated. However, experimental evidence obtained for *E. coli* and *B. subtilis* points to the involvement of a recombination-dependent concatemeric plasmid replication mechanism (2, 10, 35).

Infection of *E. coli* cells with phage T4 results in the almost immediate shutoff of host DNA replication and the breakdown of chromosomal DNA (31). Plasmid replication also stops soon after infection of pBR322-bearing cells; however, under conditions where host DNA breakdown is prevented by suitable phage mutations, plasmids can later resume replication if they share homology with the phage genome (44). Kreuzer et al. (35) identified the products of this homology-dependent mode of replication as concatemeric molecules of up to 35 tandem plasmid copies and showed that their synthesis requires the same DNA recombination genes (*uvsX*, *uvsY*, 59, 46/47, 39/52/60) known to be essential for secondary initiation during T4 late replication. In this experimental system the synthesis of concatemeric plasmid DNA is independent of the normal mode of plasmid replication.

Recently, the accumulation of plasmid concatemers after infection of *B. subtilis* with SPP1 has been analyzed (10). Accumulation started about 10 min after phage infection, simultaneously with the onset of SPP1 replication (12), and was dependent on phage-encoded (Gam-like?) function(s). Two plasmids presenting either negligible (15 bp; *−hom*) or extended (174 bp; *+hom*) DNA homology to the SPP1 genome were compared. Infection of wild-type *B. subtilis* cells bearing either plasmid resulted in similar kinetics of accumulation and final amounts of plasmid concatemers (Fig. 2). However, the plasmid transduction frequency, which provides an estimation of the amount of plasmid DNA encapsidated, was about 200-fold higher for the *+hom* plasmid (2). From these results we inferred that the rate-limiting factor determining the transduction frequency is not the amount of concatemeric DNA generated in a phage-infected cell. Subsequent experiments revealed that the difference between *+hom* and *−hom* concatemers is rather qualitative, the former being characterized by the presence of a phage-packaging signal (10) (see below).

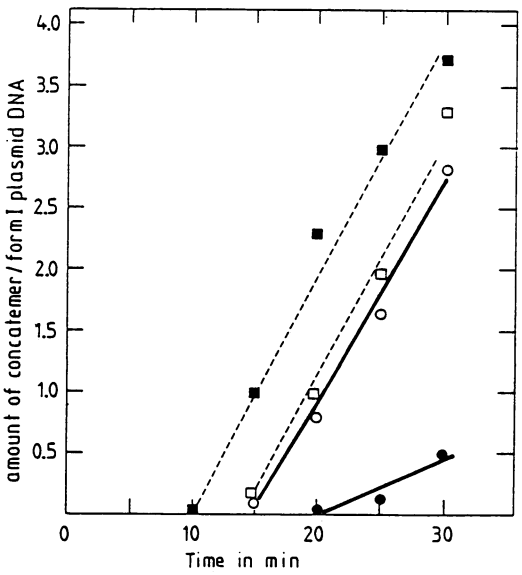

FIG. 2. Kinetics of accumulation of concatemeric plasmid DNA during SPP1 infection of wild-type and *dnaB37 B. subtilis* strains. Phage infection (time zero) was performed on exponentially growing (37°C) wild-type or *dnaB37* (46°C) cells. At 10 min before infection the *dnaB37* strain was shifted from 30 to 46°C. Plasmid pC1941 (*−hom*) or p1958 (*+hom*) crude preparations were analyzed by Southern hybridization. Relative amounts of concatemeric plasmid DNA are presented as the ratios of concatemeric to form I (monomeric and dimeric) plasmid DNA. The peaks of intensity of plasmid bands present on the autoradiograms, determined by quantitative scanning with a laser densitometer, were integrated by using the GelScan software package. The same kinetics as at 37°C were observed for both plasmids in the wild-type strain shifted from 30 to 46°C. Symbols: ○,●, plasmid pC1941 (*−hom*); □,■, plasmid p1958 (*+hom*). Open symbols, wild-type strain; solid symbols, *dnaB37* strain.

As discussed above, the mechanism of *hmw* plasmid DNA synthesis in uninfected *B. subtilis* cells relies on at least the DnaB, RecA, and PolI functions. Infection of a *B. subtilis dnaB37* strain with phage SPP1, at the nonpermissive temperature, resulted in a delayed and more than 30-fold reduced production of *−hom* plasmid concatemers, whereas amounts similar to those of the wild-type strain were observed for the *+hom* plasmid (Fig. 2). Similar data were obtained when a *recA polA* strain was used as the host (10).

These results indicated that in SPP1-infected cells two different overlapping modes of plasmid concatemeric DNA synthesis might be operative. One is a homology-independent (with the SPP1 genome), DnaB-dependent mechanism, by which *hmw* plasmid molecules, of the same nature as the ones observed in uninfected cells that are impaired in ExoV activity, are produced (see above). The other is a homology-dependent, DnaB-independent mechanism. Southern hybridization analysis of the material produced via this second mechanism revealed that it consists of phage-plasmid chimeras (10). Furthermore, the presence of one or two DNA mismatches within the 47-bp minimal region of homology reduced the plasmid-transducing frequency about 30-fold (70a).

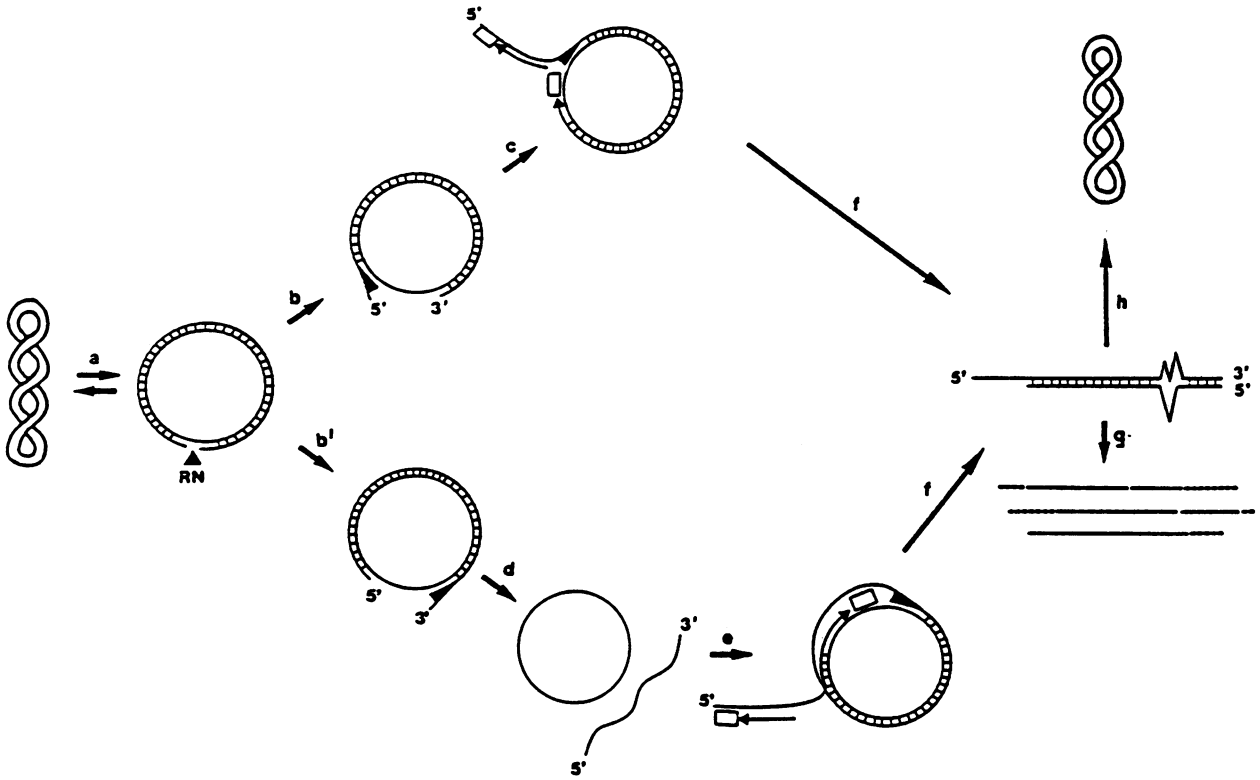

FIG. 3. Models for the generation of linear concatemeric plasmid DNA. A random nick (RN) introduced into a nonreplicative plasmid molecule (step a) is extended to a DNA gap by an exonucleolytic enzyme (PolI?). This functional gap may allow the entry of a DNA helicase with either 5'-to-3' (step b) or 3'-to-5' (step b') activity. The 3' end of the molecule in step b is used for the initiation of concatemeric plasmid DNA synthesis (step c), whereas step b' leads to the accumulation of circular and linear ssDNA molecules (step d). The single-stranded 3' end invades a homologous region of a different DNA molecule (mediated by an enzyme which catalyzes homologous pairing, e.g., RecA/UvsX), resulting in a recombination intermediate (step e). Both steps c and e may lead to the accumulation of multigenome-length plasmid molecules (step f). In the presence of an active ExoV enzyme, such linear concatemers are degraded (step g). Alternatively, concatemeric plasmid DNA molecules may be resolved into monomers (step h). Symbols: ►, DNA helicase; □, DNA PolIII. The arrows on the DNA strands indicate the 5'-to-3' polarity.

Interestingly, the absence of *−hom* plasmid linear concatemers in a SPP1-infected *recA polA* double-mutant strain was also noted. On the other hand, concatemeric DNA synthesis was unaffected in the *+hom* plasmid (10). A strong *recA* and *polA* dependence was shown for the accumulation of *hmw* plasmid material in uninfected ExoV$^-$ cells (Table 1). These observations are compatible with the conclusion that a SPP1-encoded "recombinase" function is involved in the homology-dependent, DnaB-independent mechanism. As discussed above, a similar case has been reported for the generation of plasmid concatemers in T4-infected *E. coli* cells (35).

It has been shown that SPP1 initiates unidirectional packaging on either linear or circular DNA molecules at a specific sequence, the *pac* site (11, 20). Since both the plasmid transduction frequency and the generation of phage-plasmid chimeric molecules bearing the *pac* site are phage homology dependent, we assume that such chimeras correspond to the substrate used for packaging of plasmid DNA into transducing particles, via the same mechanism as the one used to encapsidate phage DNA.

In summary, two different mechanism of plasmid concatemeric DNA synthesis may be active in phage-infected plasmid-bearing cells, as shown for the *B. subtilis*-SPP1 system (10). The two mechanisms normally act simultaneously but can be decoupled in specific DNA recombination or replication mutants. The first one is similar to the mechanism operating in uninfected cells with impaired ExoV activity and results in the production of analogous plasmid concatemers. The second one requires a significant degree of sequence homology between the plasmid and the phage genome. Unlike the first mode, it relies on a putative phage-encoded recombinase function rather than on host recombination functions. The second mechanism results in the production of a phage-plasmid chimera bearing the phage initiation packaging site, which may later be encapsidated into transducing phage particles (10, 35, 50).

MODELS FOR PLASMID CONCATEMERIC REPLICATION

The molecular events that trigger the synthesis of concatemeric plasmid molecules observed in certain genetic backgrounds, as well as in phage-infected cells, are unknown. In Fig. 3 we have depicted different possible mechanisms which accommodate previously proposed models (2, 18, 23, 25, 35, 47, 48, 51, 62). Our model can also account for the generation of plasmid multimers upon phage infection via a recombination-dependent replication mode. *hmw* DNA synthesis has been shown to be independent of the plasmid-encoded

initiation protein (see above). Therefore, we have considered in Fig. 3 the initial situation for a nonreplicating molecule (step a). The situation for replicating molecules was previously envisaged (69) and is fully compatible with both the upper (b-c-f) and lower (b'-d-e-f) pathways (see below).

A random nick is introduced into either strand of a supercoiled plasmid molecule (step a). When it is not repaired, the nick could then be converted into a gap (steps b and b') by the activity of a nonprocessive exonuclease (e.g., PolI). This functional gap could allow a DNA helicase to bind and initiate unwinding of the DNA (34). The free 3'-OH end (step b) would be elongated by DNA PolIII simultaneously with 5'-to-3' unwinding of the DNA by the helicase (step c), and plasmid rolling-circle replication could proceed (69). Alternatively, a 3'-to-5' DNA helicase (step b') could lead to the full displacement of the gapped strand (step d). The occurrence of a double-chain break (63) at any stage from step a to d can also be accommodated. The 3'-OH end of such a single-stranded plasmid molecule could then invade a double-stranded homologous region of a different DNA molecule, generating a unique recombination intermediate (step e). The invading 3'-OH end would then be used as a primer for DNA synthesis on the invaded plasmid molecule and later converted into a replication fork. DNA PolI may be involved in an early step of primer extension. A similar mechanism might be operative in the generation of plasmid multimers upon phage infection. Invasion of a double-stranded plasmid molecule by a homologous 3' single-stranded end of the phage DNA molecule may prime plasmid replication, which would then rely on phage rather than host replication functions (step e) (10, 35). Any of these mechanisms would lead to synthesis of a concatemeric plasmid molecule (step f).

In the presence of an active ExoV enzyme, the relaxed molecule (steps b and b') and/or the concatemer (step f) could be degraded (only the latter is depicted [step g]). Conversely, in the absence of ExoV or after phage infection, the generation of such plasmid concatemers would readily be revealed. Finally, the plasmid concatemers could be converted, either by intramolecular recombination (in gram-negative bacteria [64]) or via replication (in gram-positive bacteria [50]) (step h), into monomeric rings and finally the supercoiled plasmid form.

A simple way to account for the upper pathway (steps b and c) would be the case in which a unidirectionally replicating molecule fails to terminate replication as a result of further unwinding of DNA by the helicase after the normal completion of a full replication round. Consequently, the replicating complex would displace the 5' end of the newly synthesized strand. Such a displacement and subsequent elongation would result in a semiconservative rolling-circle mode of replication. This mode of replication has previously been observed in replication systems in vitro (6, 45, 46). Multigenome-length, single-stranded, linear molecules have been detected both in vivo and in vitro (23, 38, 46, 69). Alternatively, as depicted in Fig. 3, the rolling-circle mode may be initiated from random nicks introduced into supercoiled circular molecules. This is consistent with the observation that the accumulation of concatemeric plasmid DNA is independent of plasmid replication initiation functions (2, 18, 35, 44, 59, 69). *hmw* DNA synthesis is strictly dependent on an active recombinase enzyme (18, 38, 39, 69). Such a requirement, however, is not obvious for rolling-circle plasmid replication occurring as proposed in the upper (a-b-c-f) pathway (see below). Whether the latter pathway also works in vivo to generate multigenome-length plasmid molecules remains to be determined.

In the lower pathway (step e), the 3'-OH single-stranded end of a DNA molecule would prime DNA synthesis via a recombination intermediate by using a second dsDNA molecule of homologous sequence as a template (47, 62). The most striking aspects of this pathway are that: (i) no discontinuity on the DNA template is expected and (ii) it involves conservative rather than semiconservative replication. This replication mode, which has previously been observed in vitro by using a reconstituted system, was termed bubble migration (23). On the basis of (i) the requirements for the generation of plasmid multimers in both noninfected and infected cells (see above) and (ii) the fact that the formation of a phage-plasmid chimera is a prerequisite for the accumulation of packageable (*pac*-containing) plasmid concatemers in infected cells, we infer that the lower pathway would be operative in vivo (10, 39).

Finally, a similar mechanism to the one depicted in step e of Fig. 3, in which a single-stranded 3' end would be transiently extended by recombination-dependent replication on a second DNA template, could be used to remove DNA lesions or to repair double-stranded breaks (23).

CONCLUDING REMARKS

The accumulation of *hmw* DNA correlates with a reduced cell growth capacity and lethality (37, 38). Although this effect is more stringent in recombination-deficient strains, a similar phenotype was also reported for wild-type *B. subtilis* cells bearing certain recombinant plasmids which replicate via a sigma mechanism (28, 38, 69). The molecular nature of *hmw* plasmid molecules makes them potential substrates for recombination-mediated rearrangements of plasmid material (36, 51). On the other hand, recombinant plasmids interfering with the normal host physiology may exert a strong selective pressure for less deleterious clones (4a, 37, 38). The understanding of recombination-dependent plasmid replication is therefore highly relevant from a biotechnological as well as more fundamental point of view. The characterization of this plasmid replication mode was very productive and has led to attractive and testable hypotheses for further research in the field.

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