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Genetics

Specific cleavage of chromosomal and plasmid DNA strands in gram-positive and gram-negative bacteria can be detected with nucleotide resolution

(conjugational DNA transfer/rolling-circle DNA replication/relaxosome/nicking-closing activity)

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ABSTRACT **A sensitive and precise *in vitro* technique for detecting DNA strand discontinuities produced *in vivo* has been developed. The procedure, a form of runoff DNA synthesis on molecules released from lysed bacterial cells, mapped precisely the position of cleavage of the plasmid pMV158 leading strand origin in *Streptococcus pneumoniae* and the site of strand scission, *nic*, at the transfer origins of F and the F-like plasmid R1 in *Escherichia coli*. When high frequency of recombination strains of *E. coli* were examined, DNA strand discontinuities at the *nic* positions of the chromosomally integrated fertility factors were also observed. Detection of DNA strand scission at the *nic* position of F DNA in the high frequency of recombination strains, as well as in the episomal factors, was dependent on sexual expression from the transmissable element, but was independent of mating. These results imply that not only the transfer origins of extrachromosomal F and F-like fertility factors, but also the origins of stably integrated copies of these plasmids, are subject to an equilibrium of cleavage and ligation *in vivo* in the absence of DNA transfer.**

We are interested in understanding the events leading to the cleavage and initiation of DNA strand transfer of the self-transmissible, F-like, antibiotic resistance factor R1, and in clarifying the molecular mechanisms governing the initiation of DNA replication of the pMV158 family of promiscuous, rolling-circle (RC)-replicating plasmids. These systems have the common biological objective of mobilizing single-stranded (ss) DNA. Current models of plasmid leading strand replication and conjugational transfer of F-like plasmid DNA resemble each other in several mechanistic aspects (refs. 1–3 and references therein).

Plasmid pMV158 is a 5.5-kb multicopy natural promiscuous replicon that is the prototype of a family of RC-replicating plasmids isolated from several eubacteria (2). Initiation of its replication is mediated by RepB protein, which cleaves the phosphodiester bond between G-448 and A-449 of the double-strand origin (*dso*; ref. 4 and references therein). The nicking-closing activity of the purified RepB protein on various substrates *in vitro* has been examined (4, 5).

For conjugational transfer of plasmid DNA to occur, a specific plasmid strand is cleaved at a unique nick site (*nic*) within the origin of transfer (*oriT*). In keeping with present models of this process in the F system, nicking of *oriT* DNA of the closely related plasmid R1 should be catalyzed by a multienzyme complex, the relaxosome, that includes the *Escherichia coli* integration host factor protein, and plasmid-encoded TraY and TraI proteins (ref. 3 and references therein). In the R1 system, at least one additional plasmid protein, TraM, acts as an accessory DNA binding protein at *oriT* (E.L.Z., unpublished data). The site-specific DNA strand transferase activity of the relaxosome is provided by the TraI protein (6, 7).

Elegant biochemical studies have characterized the activities of relaxosomes, reconstituted *in vitro* from purified proteins, of plasmids of the F, P, and Q groups (8–11). However, the properties and regulation of these complexes at their respective transfer origins *in vivo* are less well understood. Notably, the conditions that induce initiation of conjugational DNA transfer remain obscure as does the nature of the activation. Recently, precedence was found in the R6K system for two functional *oriT*s on one plasmid molecule (12). Because transfer can initiate from only one origin, this finding reveals an additional complexity in the regulation of the start of transfer, namely, relaxosome choice.

To analyze the performance of nickases and enzyme complexes such as the R1 relaxosome *in vivo*, we have developed a methodology that permits detection of intracellular cleavage of DNA strands. This report characterizes the experimental system and presents evidence that DNA strand interruptions can be readily detected with nucleotide resolution in large (≈90 kb), very low copy number Gram-negative plasmids and in small (5 kb), RC plasmids in Gram-positive bacteria. Remarkably, *nic*-specific DNA strand discontinuities at the sites of integration of F plasmids in the chromosomes of *E. coli* high frequency recombination (Hfr) strains were also observed. From this finding, it is inferred that the transfer proteins expressed by chromosomally integrated fertility factors form functional relaxosomes that maintain an equilibrium of cleavage and joining of *nic* DNA at the point of origin of an Hfr strain even in the absence of bacterial mating.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Media. All *E. coli* strains were K12 derivatives. J5 and MC1061 were used for the maintenance and transfer of R1 and F plasmid derivatives. M1174 *sfrA*+ and M1164 *sfrA5* (13) were kindly provided by P. Silverman (Oklahoma Medical Research Foundation, Oklahoma City). The Hfr donor strains were Hfr3000 (14), KL96 (15), 61-1 *deoB-serB*Δ, and 122-1 *deoB-arcA*Δ (16). 61-1 and 122-1 were the gift of A. Dijkstra (Hoffmann–LaRoche,

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Abbreviations: Hfr, high frequency recombination; RC, rolling circle; ss, single stranded; *dso*, double-stranded origin; cfu, colony forming unit(s); ONC, overnight culture.

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Basel). W3110, LE392, and JC3272 were used for (F−) controls.

Plasmids R1*drd19* (R1-19) and R1*drd16* (R1-16) are derepressed derivatives of plasmid R1. pOX38-Km (17) was the gift of L. Frost (University of Alberta, Edmonton, Canada). pGZ119HE (18), employed for the expression of *E. coli arcA* and the R1 *traI* gene, was kindly provided by E. Lanka (Max-Planck-Institut für Molekulare Genetik, Berlin). pUAA-1 (19) was the gift of G. Sawers (University of Sussex, Brighton, U.K.). pCK217 (20) contains positions 1238–2297 of R1 DNA (numbering as in ref. 21) in pBluescript SK+ (Stratagene). pLS1 is a deletion variant of pMV158 that lacks mobilization sequences (22–24). pCGA12 (24) contains the entire *dso* of pLS1 inserted into the *Hin*dIII site of pC194 (25). pLS86 contains a 5.3-kb insertion of *Streptococcus pneumoniae* DNA including the *sulA* gene (26). The pLS1cop7 derivative of pLS1 (27) has undergone a spontaneous C to A transversion at position 743 (22). As a result, the copy number of pLS1cop7 is elevated 5-fold relative to pLS1. pCGA12, pLS86, and pLS1cop7 were maintained in *S. pneumoniae* MP551 [R6 *end-1 hexo-3 noz-19 polA*Ω*(1052*::*cat)*] (28).

The medium for *E. coli* was 2xTY (29). Media and growth of *S. pneumoniae* have been described (30). When required, antibiotics were added at the indicated concentrations (in μg ml^{-1}): kanamycin, 40; streptomycin, 25; dihydroampicillin, 50; chloramphenicol, 10 (*E. coli*) or 3 (*S. pneumoniae*); tetracycline-HCl, 1.

Oligonucleotides, Enzymes, and Reagents. The sequences of the oligonucleotides in the nicking assay and for nucleotide sequence standards were (5′ to 3′): no. 6, complementary to F factors, CCCGGCCTGCAAGATCAAAGC [R1 positions 1865–1885] and primer pMV158, used with the Gram(+) plasmids, TTCATGCGTCACCTCTC [pMV158 positions 659–643]. No. 6*, AATTGCTTTGATCTTGCAGGCCGGGAGCT was used for the control template. For the molecular cloning of *traI*, TRAI-1, TCTCCCATGCTGCCCAGCAC and TRAI-2, GACCTGCAGAAAGAGAAAACCCTGGG were used.

Restriction endonucleases, calf intestinal phosphatase, T4 DNA ligase, and T4 polynucleotide kinase were purchased from Boehringer Mannheim. Klenow enzyme was purchased from New England Biolabs. The *Thermus brockianus* thermostable DNA polymerase, DyNAoyme, was obtained from Finnzymes (Espoo). Radiochemicals were purchased from NEN.

Preparation of Expression Constructions for the *E. coli arcA* Gene and the R1 *traI* Gene. Standard recombinant DNA techniques (31) or those suggested by the manufacturers were used.

Molecular Cloning of the *traI* Gene of Plasmid R1-16. An 11.3-kb *Nsi* fragment from R1-16 DNA, identified by hybridization to 5′ and 3′ *traI* probes, was ligated to linear pBluescript SK+ DNA. Partial DNA sequence of the region upstream of *traI* of positive clone pHP1 was obtained using primer TRAI-1 and the T7 sequencing kit (Pharmacia). These data were sufficient to identify an *Asn*I site 51 nt upstream of the *traI* start codon. A 6.1-kb *Asn*I fragment of pHP1, containing *traI*, was made blunt and subcloned to *Sma*I-digested pGZ119HE DNA (P_{tac}-*lacI*^{q}). Clone pHP2 was used for complementation of nicking *in vivo*.

The *arcA* gene of *E. coli* was excised from pUAA-1 (*Eco*RI/*Hin*dIII) and introduced to pGZ119HE DNA. Positive clones were identified by PCR amplification of the *arcA* gene and complementation of conjugal donor ability of *E. coli* 122-1 *arcA*Δ.

Preparation of the Standard DNA Template. Oligonucleotide 6* was complementary to primer 6 and had additionally four nt at the 5′ and 3′ ends compatible with *Eco*RI/*Sac*I digested vector. Hybridized oligonucleotides 6* and 6 were ligated to linear pBluescript DNA, and transformed XL1 cells were selected with dihydroampicillin and 5-bromo-4-chloro-3-indolyl β-D-galactoside. One positive clone, pHP7, was purified, linearized, and reisolated for use as a standard DNA template in the nicking assay.

Conditions for PCR Amplification and for the Runoff DNA Synthesis Assay. *E. coli* donor strains for conjugation were cultured to stationary phase, then diluted 1:25 in fresh medium at 37°C. They were then grown at 37°C without shaking for one doubling (final OD_{600} 0.2–0.3). A 100-fold excess of recipients over donor cells was added directly from overnight culture (ONC) to the growing donor culture. Mating cultures were incubated at 37°C without shaking for 15–30 min. Transfer was stopped by vigorous mixing for 1 min and rapid cooling to 0°C. Plasmid-plus and plasmid-free strains not subjected to mating were cultivated as described above, and harvested at a final OD_{600} 0.1–0.6. In some experiments, ONCs were used directly. For PCR amplification of DNA, 1–5 μl of an ONC or a single colony resuspended in reaction buffer were used as template. For the runoff DNA synthesis assay, viable cell counts were obtained at harvest.

Aliquots of cultures containing 0.4–20 × 10^6 colony forming units (cfu) were centrifuged for 4–10 min at 12,000 × *g*. The medium was thoroughly removed. Cell pellets kept at 0°C were used directly (*E. coli*) or frozen on dry ice and stored at −70°C for later use (*S. pneumoniae*). For the reaction mixture, bacteria were resuspended in 25 μl ice-cold buffer containing 10 mM Tris·HCl (pH 8.8), 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 100 ng oligonucleotide, 100 μM dNTPs, and 5–10 μCi [α-^{32}P]dATP, 3,000 Ci/mmol (1 Ci = 37 GBq). For assays including F-factors, 0.5–1.0 ng standard DNA template was also present as indicated. DyNAoyme polymerase (2.5 units) was added to each mixture. The reaction was started by incubation. For experiments with F-factors, either the cycle program 95°C for 90 s/60°C for 2 min/72°C for 3 min (see Figs. 2–4) or 95°C for 90 s/60°C for 1 min/72°C for 1 min (see Figs. 5 and 6) was applied for 35 cycles. RC plasmids were treated 95°C for 90 s/60°C for 40 s/72°C for 40 s for 35 cycles. These conditions were chosen empirically by first optimizing the yield and specificity of DNA amplification from plasmid-carrying bacteria with the nicking oligonucleotide plus a second primer.

DNA products were deproteinized, recovered by ethanol precipitation, dissolved in formamide, and analyzed by electrophoresis at 40 W for 2.5–3.5 h through 6% polyacrylamide (19:1, acrylamide:bisacrylamide) gels (19.5 × 45 × 0.04 cm) containing 7 M urea. The electrophoresis buffer was 89 mM Tris borate (pH 8.3) and 2.5 mM EDTA. Gels were autoradiographed. The autoradiograms were scanned using a Umax PowerLook II system. Figures shown are the scanned images analyzed with PHOTOSHOP software and printed with an Apple LaserWriter.

Preparation of Polynucleotide Size Markers. The same primers used in the nicking assay were used to form primed DNA templates with plasmids containing the R1 *oriT* (pCK217) or the pMV158 *dso* (pLS1). The conditions for annealing and for the *in vitro* DNA sequencing reactions in the presence of 5 μCi [α-^{32}P]dATP, 3,000 Ci/mmol were as specified in the Sequenase kit for double-stranded DNA (United States Biochemical).

RESULTS

***oriT* DNA of Derepressed Plasmids Is Nicked *in Vivo* in the Absence of Conjugative Transfer.** Increasing amounts of liquid cultures containing mating *E. coli*, where the conjugative plasmid was either R1-19, which is derepressed for transfer gene expression (see Fig. 2, lanes 1–4), or pOX38-Km, a transfer-proficient derivative of the naturally derepressed F plasmid (see Fig. 2, lanes 5–8) were harvested and used in the runoff DNA synthesis assay as described (Fig. 1). An autoradiogram of the gel indicated that, when sufficient numbers of

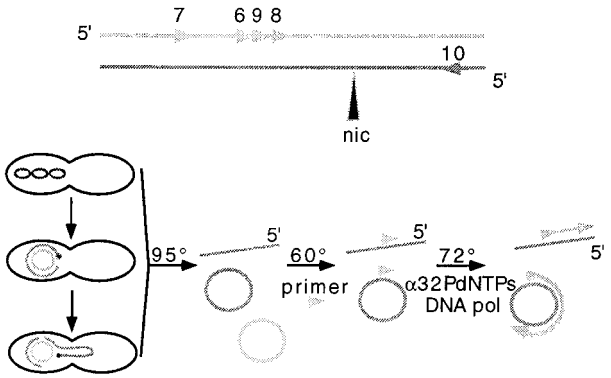

FIG. 1. Diagram of the nicking assay. The reaction is a runoff DNA synthesis with a thermostable DNA polymerase using as template ss linear or ss circular molecules, released from lysed bacteria, which have been hybridized to a single primer (triangle) 100–250 bases 3′ to the 5′ end of a suspected nick site. DNA synthesis proceeds (*Upper*, top strand) until the polymerase encounters an interruption (*nic*) in the DNA template (nicked substrates) or reaches the limits of its processivity (unnicked molecules). These unspecifically terminated reaction products can be distinguished by size from the much smaller specific termination products using a sequencing gel. The source of the specific DNA cleavage event *in vivo* could be conjugative transfer of a plasmid, as depicted (*Lower*). Cell lysis and denaturation of the released double-stranded DNA molecules (95°C), hybridization of one specific complementary DNA primer (60°C), and synthesis on primed DNA templates (72°C) are achieved in three successive incubations of the reaction mixture for 35 cycles in a thermocycler. The reaction products are labeled with [α-^{32}P]dNTP precursors. (*Upper*) Primers 6–9, complementary to the sequences of the T strand and primer 10, complementary to the retained strand, within the *oriT* of F-like plasmids.

plasmid-carrying bacteria were present in the reaction mixture, a single band of specifically terminated reaction products was apparent (Fig. 2). The DNA sequence of the size standard and

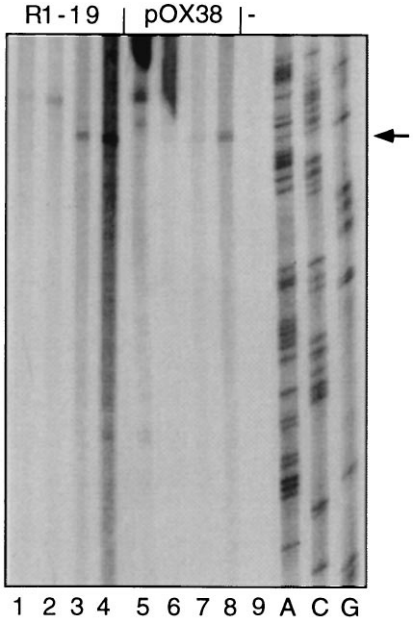

FIG. 2. The 3′ end of the *oriT*-specific reaction product maps to the same nucleotide position in plasmids R1 and F. In two separate experiments, 100, 200, 400, and 800 μl of mating cultures containing either J5[R1-19] (lanes 1–4, respectively); 50, 100, 200, and 400 μl of J5[pOX38-Km] (lanes 5–8, respectively); or 400 μl of J5 without plasmid (lane 9) were centrifuged and the cell pellets treated as described. Autoradiograms of the gels are shown. Arrow indicates reaction products terminated specifically at *nic*. Correcting for culture density, comparable numbers of cells of both strains were present in the reaction mixtures.

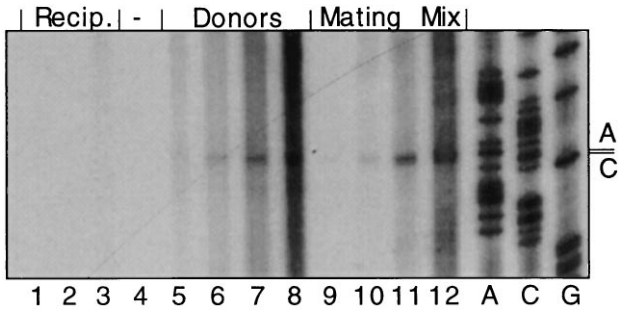

FIG. 3. The R1-19 plasmid is nicked at *oriT* in the absence of recipient cells. Culture medium containing no cells (lane 4); 60, 120, or 240 μl of recipients alone (lanes 1–3, respectively); 60, 120, 240, and 480 μl of J5[R1-19] alone (lanes 5–8); or a mating mixture of J5[R1-19] and a recipient following 15 min of incubation at 37°C (lanes 9–12) was processed for runoff DNA sequencing as described. The number of plasmid-carrying cells in reaction mixtures containing either donor alone or the mating mixture was the same. The position of *oriT*-terminated reaction products is indicated by the R1 nucleotides C-2040 and A-2041 (numbering as in ref. 21), which complement the nick site within the consensus 3′-TCGTGG ↓ TGTGG-5′ (3).

of the reaction products were identical. In accordance with the identity of the DNA sequence of both the R1 and F plasmids between the site of complementation of the primer and the expected *nic* site (32–34), specifically terminated reaction products corresponding in size to the distance separating the primer from *nic* were generated from both conjugative plasmids. This places the site of intracellular cleavage at the R1 *oriT* between position G 2040 and T 2041 (numbering as in ref. 21).

Specific termination of DNA synthesis at this site within the R1 *oriT* was not dependent on the oligonucleotide used as primer. DNA synthesis initiated from three additional oligonucleotides (Fig. 1, nos. 7, 8, and 9), which anneal to the transferred strand upstream from the expected *nic* site, led to site-specific termination at this position (not shown). In agreement with the strand-specificity of the nicking reaction *in vivo*, synthesis from a primer complementary to the opposite strand (Fig. 1, no. 10) did not terminate at this position (not shown).¶

To determine whether formation of the specific *nic*-terminated reaction product was dependent on conjugal transfer of the plasmid, samples of separate cultures of plasmid-free *E. coli* MC1061 and *E. coli* J5[R1-19] were compared in the nicking assay with aliquots of a mixed culture of these two strains undergoing mating (Fig. 3). No specific reaction product was formed in reaction mixtures without bacteria (lane 4) or in those containing plasmid-free bacteria (lanes 1–3). Reaction products terminated specifically at *nic* were observed when the culture of mating cells was tested (lanes 9–12), as well as when *E. coli* J5[R1-19], which was grown in the absence of a recipient strain, was sampled (lanes 5–8). Formation of this unique product was dependent on the presence of plasmid but was independent of conjugal transfer. The specific *in vitro* DNA reaction products observed here and in all subsequent experiments appear to migrate as two bands that differ in length by one nt. We expect that formation of two specific products was due to the terminal transferase activity of the

¶Two types of artefacts were formed routinely in these experiments when too few bacteria were used relative to the amount of primer present in the reaction mixtures (Fig. 2, lanes 1, 2, 5, and 6). The elongated smears were common to several different oligonucleotide primers when used in excess in the assay and may result from primer–primer interactions. The specific band approximately 10 bases longer than the reaction products terminated at *nic*, which was also present at high primer to cell ratios, was reproducible and unique to the particular primer used (Fig. 2, lanes 1, 2, 5, and 6; Fig. 4, lanes 6 and 7).

DNA polymerase and not to the presence of two independent cleavage sites in the plasmid DNA.

Synthesis of *nic*-Terminated DNA *in Vitro* Is Dependent on Transfer Gene Expression from the R1 Plasmid *in Vivo*. The enzyme that relaxes the transfer origin of F factors, TraI, is plasmid encoded. To ascertain whether formation of the *nic*-specific *in vitro* reaction product was dependent on transfer gene expression, separate cultures of *E. coli* J5 carrying two R1 plasmid derivatives that exhibit different levels of transfer gene expression were compared in the nicking assay (Fig. 4). The wild-type R1 plasmid is subject to fertility inhibition (*fin*+) and, as a result, transfers 1000-fold less frequently than the *fin*− R1-19 derivative. Although comparable numbers of plasmid-carrying bacteria were assayed, *nic*-terminated reaction products were not observed with the *fin*+ R1 plasmid (lanes 2–5), but were readily detected when reaction mixtures contained at least 2.7×10^6 viable cells carrying the R1-19 plasmid, which produces its transfer proteins constitutively (lanes 6–9).

To be able to monitor the efficiency of the DNA polymerization, as well as the recovery from additional handling of the reaction products, a DNA template was created for use as an internal control. Briefly, the sequence of primer 6, specific for the *oriT* region of F-like plasmids, was inserted in an unrelated plasmid vector. This DNA was isolated and cleaved once with a restriction endonuclease. When introduced as purified DNA to the reaction mixture of the nicking assay, together with the same oligonucleotide primer, this DNA template yields a second reaction product of defined length (194 nt) that is distinguishable in size from those produced on nicked molecules of the conjugative plasmids (176 nt). Following optimization, 0.5–1 ng of purified standard DNA template was chosen for routine use in the assay (not shown). Presence of the second DNA template in the reaction mixture did not inhibit synthesis from the R1-16 plasmid DNA. However, aberrant products were formed when excessive amounts of the standard DNA were used.

The results shown in Fig. 4 suggested strongly that formation of *nic*-terminated reaction products *in vitro* were dependent on transfer gene expression from the plasmid *in vivo*. Despite the fact that the transfer origins of plasmids R1 and R1-19 are identical, the comparison is still one of two distinct plasmids. Therefore, we sought to corroborate this evidence with another approach and additionally include the internal control in the assay. For this experiment, the same R1 plasmid derivative was now compared in two different host strains that individually support different levels of transfer gene expression from the plasmid. *E. coli* M1174 and M1164 are isogenic strains that differ by a V203M mutation in the *arcA* gene of M1164 (P. Silverman, unpublished data). *E. coli arcA*, also known as *sfrA* (sex factor regulator A) and *fex* (F expression) encodes the regulator element of a two-component signal transduction system, which is known to positively regulate sexual expression from F factors (13, 35). Ten-fold lower levels of expression from the main transfer operon promoter of F were found in M1164 *sfrA5* than in the wild-type M1174 strain (36). To determine whether the specific *in vitro* reaction product we observed was dependent on the presence of *arcA* and thus required sexual expression *in vivo*, overnight cultures of R1-16-carrying M1174 and M1164 were compared in the nicking assay (Fig. 5, lanes 1–4). Synthetic products terminated at the position of *nic* were apparent only in reaction mixtures when the R1-16 plasmid was maintained in the wild-type *arcA* strain. Formation of this product *in vitro* was complemented by the coresidency in M1164 *sfrA5*[R1-16] of a second plasmid capable of expressing in *trans* the *E. coli arcA* gene (lanes 5 and 6), or the *traI* gene of plasmid R1 (lanes 7 and 8). Coresidency of the expression vector alone was not responsible for this effect. Overexpression from the LacI-regulated *tac* promoter was not induced chemically in this experiment. Complementation of the nicking activity mediated by *traI* in *trans* was notably weaker than when *arcA* was present. This difference results from the ability of *arcA* to complement transfer gene expression to wild-type levels under these conditions (G. Koraimann and E.L.Z., unpublished data), whereas coresidency of a *traI*-carrying plasmid provides TraI alone. In this case, the reduced abundance of the accessory DNA binding proteins seems to limit the efficiency of the nicking reaction *in vivo*.

As presence of either the *arcA* gene or the *traI* gene of R1 in *trans* to the R1-16 plasmid in the M1164 strain complemented formation of the *nic*-specific reaction product *in vitro*, we conclude that formation of this product was dependent on transfer gene expression *in vivo*. These results, taken together, established that the *nic*-specific reaction product was generated from plasmid DNA, which was nicked *in vivo* at the transfer origin.

The F Transfer Origins in Hfr Strains Are Subject to Transfer-Independent Cleaving-Joining Activity *in Vivo*. Hfr donor strains emerge from the stable integration of a transfer-proficient plasmid into the chromosome. This study has shown that nicked T strands of R1 derivatives were readily detectable in *E. coli* if their transfer proteins were produced constitutively (Fig. 4). *Tra* gene expression from the F plasmid is naturally derepressed (*finO*−). Therefore, we wondered whether the chromosomally integrated copies of the F plasmids in Hfr strains would similarly experience transfer-independent cleaving-joining activity at their transfer origins. To investigate this possibility two Hfr strains, Hfr3000 and KL96, and three female (F−) strains were analyzed in the nicking assay using *oriT*-specific primer 6 for F factors (Fig. 6*A*).

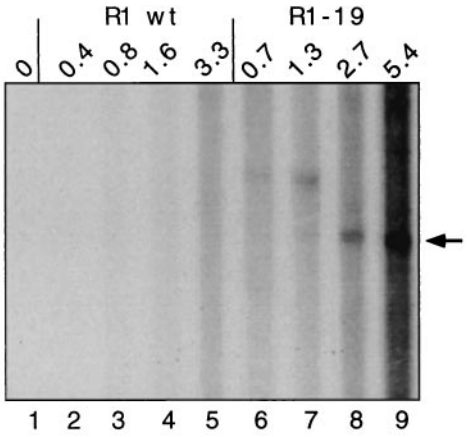

FIG. 4. Synthesis of *nic*-terminated DNA *in vitro* was observed from R1-19 (*finO*−) but not the fertility-inhibited R1 plasmid. Medium (lane 1), increasing amounts of cultures containing J5[R1] (lanes 2–5), or J5[R1-19] (lanes 6–9) were prepared for the DNA synthesis assay. Arrow indicates position of *nic*-specific reaction products. The number of viable cells present in each reaction mixture is indicated above the lanes (cfu $\times 10^6$).

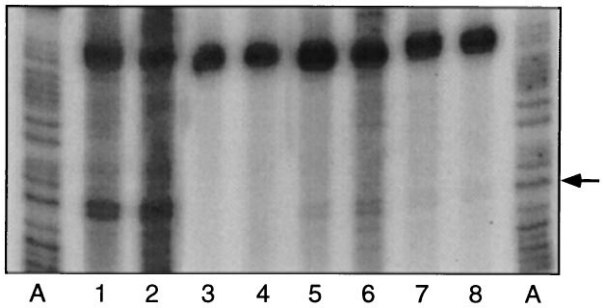

FIG. 5. Synthesis of *nic*-terminated DNA *in vitro* requires transfer gene expression from the R1 plasmid *in vivo*. Reaction mixtures containing M1174[R1-16+pGZ119] (lanes 1 and 2), M1164[R1-16+pGZ119] (lanes 3 and 4), M1164[R1-16+pGZarcA] (lanes 5 and 6), or M1164[R1-16+pHP2] (lanes 7 and 8) were subjected to runoff nucleotide synthesis. The numbers of viable cells analyzed in lanes 1–8 were, respectively, 2.3, 4.6, 7.8, 15.6, 8.8, 17.6, 5.6, and 11.2 ($\times 10^6$ cfu).

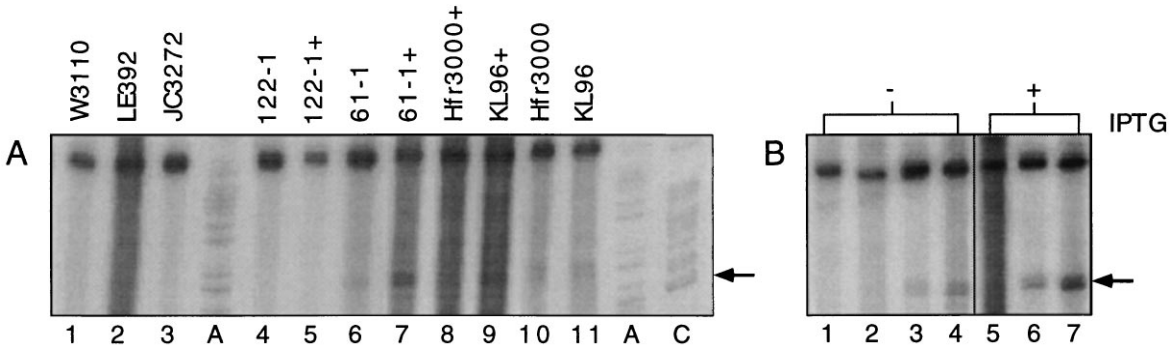

FIG. 6. Detection of transfer-independent cleavage activity at the *oriT*s of Hfr strains requires synthesis of F transfer proteins *in vivo*. (*A*) Forty microliters from ONCs of three F(−) strains (lanes 1–3) and four Hfr strains without (lanes 4, 6, 10, and 11) and with (+) the R1-16 plasmid (lanes 5, 7, 8, and 9) were prepared for a runoff nucleotide sequencing assay. (*B*) Forty and 80 μl of ONCs of 122-1[pGZ119] (lanes 1 and 2) and 122-1[pGZarcA] (lanes 3 and 4), or aliquots of cultures of 122-1[pGZ119] (lane 5) or 122-1[pGZarcA] (lanes 6 and 7), which were diluted first in fresh medium containing 10 mM isopropyl β-D-thiogalactoside and incubated with shaking at 37°C for 2.5 h, were prepared for the nicking assay. The numbers of viable cells analyzed in the reaction mixtures in lanes 1–7 were, respectively, 0.65, 1.3, 30.8, 61.5, 22.1, 0.09, and 0.15 ($\times 10^6$ cfu). The *nic*-specific reaction products are indicated by arrows.

Two additional HfrH strains, 61-1 *deoB-serB*Δ and 122-1 *deoB-arcA*Δ, were also examined to assess simultaneously the dependence of the reaction products on gene expression from the F factors *in vivo*. *In vitro* DNA synthesis from the standard DNA template was essentially equivalent in all reaction mixtures (upper band). A second band, migrating at the position expected for *nic*-terminated reaction products from the F plasmid (arrow), was observed in each reaction including an Hfr strain (lanes 6, 10, and 11), except for Δ*arcA* strain 122-1 (lane 4), and was absent from those containing an F(−) strain (lanes 1–3). When the Hfr strains contained additionally R1-16, a band with the same apparent mobility and increased intensity was observed (lanes 7–9), suggesting that both the R1 and F *oriT*s served as a DNA template for the *in vitro* DNA synthesis. A band of this size was not generated from the HfrH Δ*arcA* strain without (lane 4) or with plasmid R1-16 (lane 5).

If initiation of synthesis of this reaction product took place on the primed origin DNA of F plasmids linked to the bacterial chromosome and, if termination of the *in vitro* reaction occurred at the 5′ ends of the T strands cleaved *in vivo* by F-determined relaxase, then it should be possible to complement formation of this DNA product in the 122-1 HfrH Δ*arcA* strain by complementing positive regulation of gene expression from F. An expression construction, pGZarcA, with the *E. coli arcA* gene under P_{tac} control and vector alone, were introduced to 122-1. Reaction products from noninduced and isopropyl β-D-thiogalactoside-induced cultures of these strains were compared in the nicking assay (Fig. 6*B*). A reaction product corresponding in size to *nic*-specific termination *in vitro* was obtained in 122-1 when the *arcA* gene was provided on a plasmid. Induction of *arcA* expression resulted in a higher yield of the unique *nic* product despite a more than 300-fold reduction in the number of cells analyzed under conditions of overexpression. Given this result, we infer that the DNA strand scissions detected in these experiments with Hfr donor strains reflect the cleaving-joining activity of relaxosomes positioned at the *oriT*s of the integrated F factors.

Identification of the Site of Initiation of Plasmid pMV158 Leading Strand Replication in *S. pneumoniae*. To determine whether the cleavage ability exhibited by the purified replication initiator protein, RepB, on *dso*-containing DNA substrates *in vitro* reflects properly the performance of the enzyme in initiating DNA replication *in vivo*, linear replication intermediates of pMV158 derivatives were released from *S. pneumoniae* and analyzed in the nicking assay (Fig. 7). A unique band corresponding in size to the distance separating the primer and the site of *in vitro* initiation, *nic*, was observed when *S. pneumoniae* carried a pLS1 derivative encoding wild-type replication elements (lane 1) or a copy-up mutant (lane 2), but not when the pLS1 *dso* alone was present heterologously on a different RC replicon (lane 3). In agreement with the elevated copy number of pLS1cop7 compared with wild-type pLS1, the abundance of the *nic*-terminated reaction product generated from the pLS1cop7 mutant was greater than that from pLS86 when equivalent numbers of bacteria were analyzed.

DISCUSSION

Conjugative plasmids play an important role in genome plasticity by mediating horizontal gene transfer. Dispersal of genetic material occurs via plasmid self-transfer as well as through mobilization of nonconjugative plasmids and chromosomal genes. The identification and characterization of naturally occurring replicons, therefore, will continue to receive attention in bacterial genetics. The localization of Tra, Mob, and replication origins belongs to the basic criteria with which new plasmids and conjugative systems are analyzed. The presented methodology was shown to be well suited to mapping discontinuities in DNA strands generated *in vivo*, and to locate precisely cleavage sites not previously mapped, such as the *nic* site of plasmid R1 (Fig. 2). The technique should prove particularly useful for fine mapping in new systems once an origin-containing restriction fragment has been identified. This feature is important, since conventional analyses of an *oriT* DNA fragment, for example, can reveal several putative origins, any one or more of which may be active *in vivo* (37). It is an advantage that this procedure is as effective in Gram-positive as in Gram-negative bacteria. In some instances, the characterization of origins from Gram-positive systems must be carried out in surrogate Gram-negative hosts because properties of the natural host preclude isolation of relaxation complexes (38). In this context, it is also significant that the application

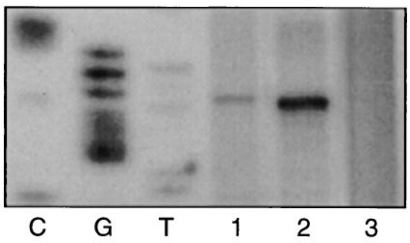

FIG. 7. Leading strand replication of pMV158 derivatives initiates at the same site in *S. pneumoniae* that is cleaved by RepB protein *in vitro*. Bacteria were cultured to OD_{600} 0.25–0.3. Aliquots containing 4×10^6 cfu of *S. pneumoniae* strains MP551[pLS86] (lane 1), MP551[pLS1cop7] (lane 2), and MP551[pCGA12] (lane 3) were subjected to runoff DNA synthesis, as described, except that 10 ng of primer pMV158 was present in each reaction mixture.

of this methodology was not limited by low plasmid copy number nor by the size of the DNA substrate. In addition, proposed nick sites within the *dso* of RC plasmids can now be unambiguously defined. As shown in this study (Fig. 7), the procedure can be used to confirm whether the position of strand scission observed *in vitro* properly reflects the position of bond breakage catalyzed *in vivo*.

The formation of DNA–protein relaxation complexes is a general feature of plasmids of *E. coli* (39), first observed with ColE1 by Clewell and Helinski (40), and subsequently found for a variety of conjugative and mobilizable plasmids (41). Since the initial observation, the issue of whether or not the supercoiled DNA of nucleoprotein relaxation complexes, which is released in a relaxed form following treatment with heat or protein denaturing agents, is actually maintained in an equilibrium of nicking and closing *in vivo*, or whether cleavage is simply induced by the treatment *in vitro*, has not been resolved unequivocally. Several lines of evidence exist to support the view that relaxosomes maintain an equilibrium of cleaving and joining activity *in vivo*. Compelling evidence is available from IncW, IncF, and IncQ plasmids, where enhanced recombination of *oriT*-containing DNA *in vivo* has been observed that required additionally the cognate cleaving–joining activity but was independent of conjugative transfer (42–44). Additional evidence stems from the well-characterized *in vitro* properties of the relaxosomal proteins of the IncP plasmid RP4. The key component of this multienzyme complex, TraI, has been shown to possess cleaving–joining activity, resembling a type I topoisomerase (45). Cleavage of ss oligonucleotides by TraI does not require induction by a protein denaturing agent. A mutant form of TraI (S74A) was also shown to act together with the TraJ protein to convert form I *oriT* DNA to partially relaxed covalently closed plasmid forms (46). The partial relaxation took place in the absence of denaturing agents, therefore, cleaving–joining of the plasmid DNA must occur continuously and not only when the complex is disrupted. Based on these *in vivo* and *in vitro* data, we assume that relaxosomes sustain an equilibrium of nicking and closing of *nic* DNA *in vivo* without the stimulus of conjugation. This report has been written based on this premise.

In this study, the specificity of the products of the nicking assay with F factors was demonstrated according to several criteria (Figs. 2–5). Based on the premise stated above, it follows, therefore, that the transfer protein-dependent formation of the same specific reaction products from the Hfr donor strains (Fig. 6) was due to the cleaving–joining activity exerted by relaxosomes positioned at the *oriT*s of the integrated fertility factors.

The strength of this approach lies in the ability to observe directly the position and frequency of DNA processing reactions that occur *in vivo* with substrate and enzyme in their natural context. This technique could, therefore, prove useful for monitoring a variety of DNA transactions producing intermediates with DNA strand discontinuities. This could include initiation, termination, and paused intermediates of DNA replication, as well as intermediates of site-specific recombination, transposition, and perhaps viral integration. Extension of the technique to studies with eukaryotes is feasible but remains to be tested. The next step is to address questions about the concerted mechanisms that affect the replicative and conjugative DNA processing reactions observed here.

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