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Single-stranded plasmid DNA in *Bacillus subtilis* and *Staphylococcus aureus*

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ABSTRACT **Plasmid pC194 was found to exist in a double-stranded and a single-stranded DNA form in *Bacillus subtilis* and *Staphylococcus aureus*. This single-stranded DNA was found as a circular molecule of the same size as the parental monomer and corresponded to only one of the two DNA strands. It represented one-third of plasmid copies. Single- and double-stranded DNA copies in similar proportions to the above were detected for five other *S. aureus* plasmids (pC221, pC223, pE194, pT127, and pT181) and one *B. subtilis* plasmid (pHV416). *S. aureus* plasmid pUB110 and *Bacillus cereus* plasmid pBC16 were, in contrast, predominantly double-stranded.**

Many small plasmids conferring resistance to different antibiotics have been isolated from *Staphylococcus aureus* (1, 2). One of the striking features of this group of plasmids is their broad host range. Thus, some *S. aureus* plasmids replicate not only in another Gram-positive bacterium, *Bacillus subtilis*, a capability that opened the possibility of their use as cloning vectors in this host (3, 4), but also in the Gram-negative bacterium *Escherichia coli* (5). Furthermore, plasmids pC194, pC221, and pC223 were found to be maintained extrachromosomally in the yeast *Saccharomyces cerevisiae* (6). Transfer of these plasmids among different bacterial species probably occurs in nature, as deduced from the observation that plasmid pBC16 isolated from *Bacillus cereus* (7) has a replication region very similar to that of the *S. aureus* plasmid pUB110 (8).

Plasmids pE194 (9), pC194 (10), pC221 (11, 12), and pT181 (13) have been fully sequenced, which made possible a comparison of their genetic organization. All four plasmids seem to encode a specific replication initiator protein, called Rep, and contain an origin sequence within or near the gene specifying the N-terminal region of the Rep protein (12, 14, 15). In this paper we demonstrate that all these and several other *S. aureus* plasmids are present intracellularly not only as double-stranded DNA but also as single-stranded circular DNA.

MATERIALS AND METHODS

Bacteria and Plasmids. *B. subtilis* strain HVS49 (16) was used as a host for *S. aureus* plasmids pC194, pE194, pE194 *cop-6* [a copy mutant of pE194 (17)], pUB110, pT127, pC221, and pC223, *B. cereus* plasmid pBC16 (7), and *B. subtilis* replicon pHV416 (16). *S. aureus* strains RN2425 (pC194), RN2442 (pE194), RN3732 (pE194 *cop-6*), RN3214 (pUB110), RN2888 (pT127), RN3259 (pT181), RN5091 (pC221), and RN1754 (pC223) were a gift of S. Projan.

Growth Conditions. *B. subtilis* and *S. aureus* were grown in LB medium (19) and brain/heart infusion (Difco), respectively, supplemented with the appropriate antibiotics.

Preparation of Cell Lysates and Gel Electrophoresis. Growing cells were harvested at $OD_{650} \approx 1$, washed with EDTA (0.1 M, pH 8)/NaCl (0.15 M), and resuspended in EDTA (0.01 M, pH 6.9)/NaCl (0.15 M). Lysozyme at 10 mg/ml (for *B. subtilis*) or lysostaphin at 50 μg/ml (for *S. aureus*) was added, and, after 15 min of incubation at 37°C, lysis was completed by the addition of Sarkosyl to 1%. Lysates were heated at 65°C for 20 min and extracted with phenol and chloroform. Finally, the lysates were incubated for 20 min at 37°C in the presence of RNase at 50 μg/ml. For nuclease S1 treatment, lysates were adjusted to 30 mM sodium acetate, pH 4.6/0.5 mM $ZnCl_2$/250 mM NaCl and incubated for 45 min at 37°C in the presence of nuclease S1 at 100 units/ml.

DNA was electrophoresed at 2.5 V/cm through 0.8% agarose gels in Tris/acetate buffer containing ethidium bromide at 0.5 μg/ml.

Transfer of DNA to Nitrocellulose and Hybridization. DNA was transferred from the gels to nitrocellulose by diffusion (18) using 10× NaCl/Cit (1× NaCl/Cit is 0.15 M NaCl/0.015 M sodium citrate). In general, the DNA was not denatured prior to transfer.

Radioactive probes were prepared either by nick-translation of purified plasmid DNA in the presence of [α-^{32}P]dCTP or by end-labeling of single-stranded bacteriophage DNA in the presence of [γ-^{32}P]rATP (19). Bacteriophage R208, consisting of f1 phage DNA and pBR322, was a gift of N. D. Zinder (20). From this phage, another phage, R802, was constructed, which consists of f1 DNA and pBR322 DNA linked in the opposite orientation. Single-stranded phage DNA was extracted from phage particles as described by Zinder and Boeke (21) and purified by hydroxyapatite chromatography. Prior to ^{32}P end labeling, circular single-stranded phage DNA was mildly digested with DNase I and treated with alkaline phosphatase.

Filters were hybridized in 5× NaCl/Cit/0.5% sodium dodecyl sulfate/50% (vol/vol) formamide at 37°C for 20 hr, washed in 2× NaCl/Cit, and exposed to Fuji x-ray films. The density of bands was measured with a Shimadzu densitometer.

Purification of Single-Stranded DNA and Electron Microscopy. A lysate was applied to a hydroxyapatite column equilibrated with 75 mM sodium phosphate buffer, pH 6.8. Single-stranded DNA was eluted by a gradient ranging from 75 to 250 mM phosphate buffer. Samples were spread by the formamide technique of Westmoreland *et al.* (22). Grids were stained with uranyl acetate, rotary shadowed with platinium, and examined with a Philips 410 electron microscope. For size measurements, single-stranded DNA extracted from a

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Abbreviations: H and L, heavy and light strands of pBR322 DNA.
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hybrid phage composed of f1 and pC194 was used as an internal standard.

RESULTS

Detection of Single-Stranded Plasmid DNA. A lysate prepared from *B. subtilis* cells harboring pC194 was electrophoresed through an agarose gel and stained with ethidium bromide. A plasmid band at the position expected for pC194 supercoiled monomers could be seen (Fig. 1*A*, lane a). The DNA was denatured, transferred to nitrocellulose, and hybridized with ^{32}P-labeled pC194 DNA. Hybridization was detected at positions corresponding to supercoiled and open circular plasmid monomers. In addition, a band was visible that migrated faster than supercoiled monomers and represented about 20% of the total radioactivity, as judged by densitometric analysis of the autoradiograph (Fig. 1*B*, lane a).

The same DNA preparation was treated with nuclease S1 and electrophoresed. Ethidium bromide staining revealed bands at the positions of supercoiled plasmid monomers and nicked monomers (Fig. 1*A*, lane b). Both plasmid forms were detected by hybridization, but the fast-migrating band had disappeared (Fig. 1*B*, lane b).

The same DNA preparation, either treated or not treated with nuclease S1, was transferred to nitrocellulose without prior denaturation and hybridized with labeled pC194. Only the fast-migrating band was visible in the sample not treated with nuclease S1 (Fig. 1*C*, lane a); it had disappeared in the treated sample (Fig. 1*C*; lane b). When a lysate was prepared from plasmidless *B. subtilis* cells, no plasmid band was visible on the ethidium bromide stained gel, and no hybridization was detected (not shown).

These results show that, in lysates prepared from *B. subtilis* cells harboring pC194, sequences homologous to pC194 are present that (*i*) migrate faster than supercoiled monomeric plasmid DNA, (*ii*) are sensitive to nuclease S1, and (*iii*) bind to nitrocellulose. We conclude, therefore, that some of the plasmid DNA was present as single-stranded monomers.

Similar results were obtained with plasmid pHV50, which consists of pC194 and the ampicillin resistance gene of pBR322 (Fig. 1, lanes c and d). The only difference was that hybridization was also detected at the position of chromosomal DNA in the sample denatured before transfer (Fig. 1*B*). We have no explanation for this observation.

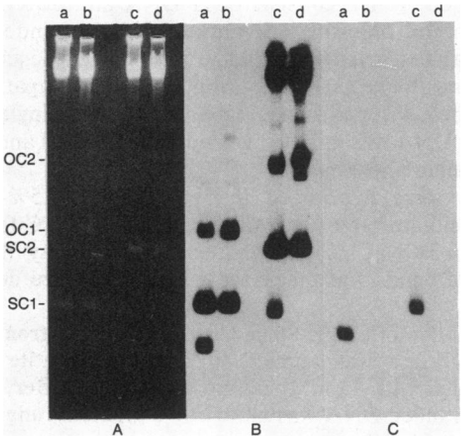

FIG. 1. Detection of single-stranded pC194 DNA and pHV50 DNA. (*A*) Agarose gel electrophoresis of lysates prepared from *B. subtilis* containing pC194 (lanes a and b) or pHV50 (lanes c and d). Samples were treated (lanes b and d) or not treated (lanes a and c) with nuclease S1. The DNA was denatured (*B*) or not (*C*) prior to transfer to nitrocellulose and was then hybridized to ^{32}P-labeled pC194. SC and OC refer to the positions of supercoiled and relaxed monomers of pC194 (1) and pHV50 (2).

Characterization of Single-Stranded Plasmid DNA. Single-stranded pC194 DNA was purified by hydroxyapatite chromatography and visualized by electron microscopy. Circular molecules were detected (Fig. 2). Their contour length determined by comparison with an internal standard (single-stranded f1–pC194 hybrid phage DNA) was 2.9 ± 0.3 kilobases (mean ± SD; 45 molecules were measured), which corresponds to the size of the plasmid monomer [2906 base pairs (15)].

Is circular single-stranded plasmid DNA generated from one or from both pC194 strands? To answer this question we used two plasmids, pHV50 and pHV51, composed of pC194 and the pBR322 ampicillin resistance gene. The relative orientations of the pC194 and pBR322 sequences are different in pHV50 and pHV51: The pC194 strand that carries the sense message for the protein A (the A-protein-coding strand) is linked to the pBR322 heavy (H) strand in the former plasmid, and to the light (L) strand in the latter (Fig. 3). Two probes specific for the two pBR322 strands were obtained from hybrids between pBR322 and the single-stranded DNA phage f1, named R208 (20) and R802, which differ in the relative orientation of the two parental genomes: the L strand of pBR322 is present in R208 phage particles, and the H strand is present in R802 phage particles.

DNA was extracted from cells carrying pHV50 and pHV51, electrophoresed, transferred to nitrocellulose without denaturation in order to bind only single-stranded DNA, and hybridized to pBR322, R208, or R802 DNA. The pBR322 DNA was labeled by nick-translation, whilst the other two, which were isolated from phage particles, were partially cleaved with pancreatic DNase, then treated with alkaline phosphatase, and finally end-labeled with polynucleotide kinase. The results are shown in Fig. 4. The first probe, containing both of the pBR322 strands, hybridized to pHV50 and pHV51 DNA. The R208 probe, containing the pBR322 L strand, hybridized to pHV50 single-stranded DNA; the R802 probe, containing the H strand, hybridized to pHV51 single-stranded DNA. This result shows that the H strand of pBR322 was single-stranded in pHV50 and the L strand in pHV51, which is expected if only the pC194 strand that carries the message for protein A (broken line, Fig. 3) is rendered single stranded.

Plasmids Other Than pC194. To determine whether plasmids other than pC194 generate single-stranded DNA, we examined extracts prepared from *B. subtilis* cells harboring *S. aureus* plasmids pE194, pE194 *cop-6*, pT127, pUB110, pC221, and pC223, the *B. subtilis* plasmid pHV416 (constructed by inserting the pT127 tetracycline resistance gene in the cryptic plasmid pHV400), and the *B. cereus* plasmid pBC16. Fast-migrating DNA sensitive to nuclease S1 and binding to nitrocellulose without denaturation was detected by hybridization with probes obtained by labeling the corresponding plasmids (Fig. 5). The amounts of single-stranded DNA relative to double-stranded DNA (visualized on agarose gels, not shown) varied not more than 2- to 3-fold for five of the plasmids (pE194, pT127, pC221, pC223, and pHV416); this variation may not be significant. pE194 *cop-6*, which has a 10 times higher number of double-stranded copies than pE194, was not present in more single-stranded copies than pE194. Almost no single-stranded DNA was detected in cells harboring *S. aureus* plasmid pUB110 or *B. cereus* plasmid pBC16. The two plasmids are known to have a very similar replication region (8). These results indicate that, in the case of the plasmids studied here, the generation of single-stranded DNA in *B. subtilis* is the rule rather than the exception. A low amount of a slower-migrating, nuclease S1-sensitive DNA was detected in several cell extracts

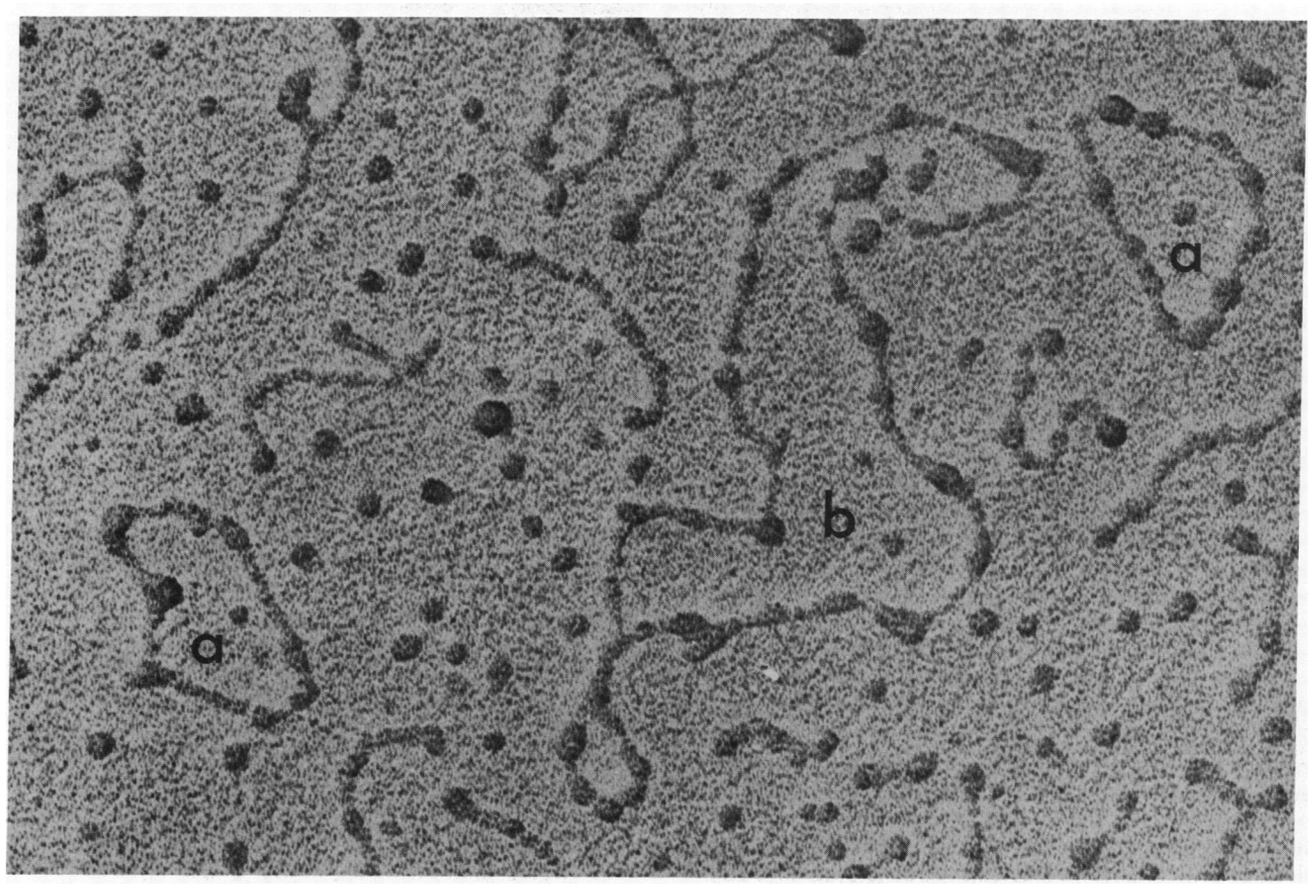

FIG. 2. Single-stranded pC194 DNA. Single-stranded pC194 DNA was purified by hydroxyapatite chromatography and visualized by electron microscopy. a, pC194 DNA; b, f1–pC194 hybrid phage DNA, the internal standard.

(pC194, pE194, pT127, and pC223) (Fig. 5). Possibly this corresponds to single-stranded dimeric plasmids.

To investigate whether *S. aureus* plasmids generate single-stranded DNA in their natural host we analyzed DNA extracts prepared from *S. aureus* cells. In all cases single-stranded DNA was detected (Fig. 6). For plasmids pE194 and pE194 *cop-6*, or pT181 and pT127 [the last two plasmids are very similar, differing by a nucleotide substitution in the region involved in copy number control (ref. 23; unpublished data)] as well as for pC194 and pC221 the amount of hybridization was roughly proportional to the double-stranded plasmid copy number, as evaluated by visual inspection of the ethidium bromide-stained agarose gels (not shown). pUB110, and perhaps pC223, generated much less single-stranded DNA per double-stranded genome than the other plasmids (Fig. 6).

The ratio of single-stranded to double-stranded DNA in *B. subtilis* is similar to that in *S. aureus* for most plasmids, with the exception of pE194 *cop-6*, and pC223. The former generated more, the latter less single-stranded DNA in *S. aureus* than in *B. subtilis*, respectively. This indicates that for these two plasmids either the rate of production or the stability of single-stranded DNA may be different in the two hosts.

DISCUSSION

The experiments described in this report show that single-stranded DNA can be detected in extracts of *B. subtilis* and *S. aureus* cells harboring certain plasmids. Several observa-

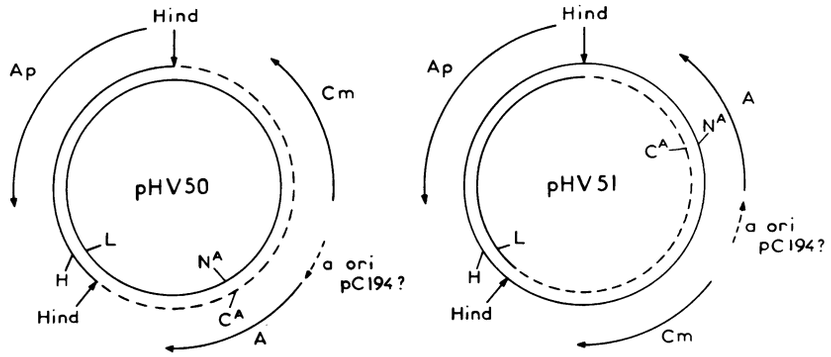

FIG. 3. Schematic representations of plasmids pHV50 and pHV51. H and L refer to the heavy and light strands of pBR322, respectively. C^A and N^A are the coding strand and the noncoding strand of the pC194 A protein. a and A refer to the site and the protein needed for pC194 replication (15). Ap, ampicillin resistance; Cm, chloramphenicol resistance; ori, origin.

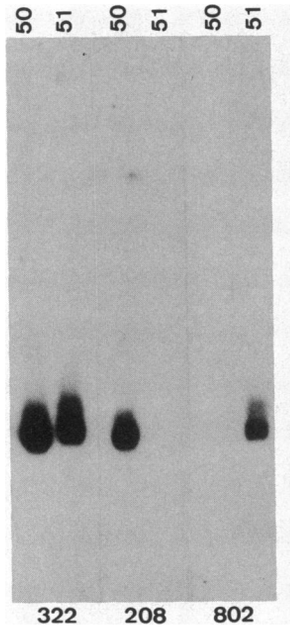

FIG. 4. Southern hybridization of pHV50 (50) and pHV51 (51) single-stranded DNA. DNA prepared from pHV50- and pHV51-containing *B. subtilis* was run through an agarose gel and transferred to nitrocellulose. Filters were hybridized to ^{32}P-labeled pBR322 DNA, ^{32}P end-labeled single-stranded phage R208 DNA, and ^{32}P end-labeled single-stranded phage R802 DNA.

tions rule out the possibility that this DNA was produced during purification by a step causing denaturation of duplex DNA. (*i*) Only one strand of pC194 was single stranded. Denaturation would render both strands single. (*ii*) Plasmid pE194 *cop-6* is maintained in *B. subtilis* at a 10-fold higher copy number than pE194, probably due to a single base pair change (17). Nevertheless, we detected more single-stranded pE194 DNA than single-stranded pE194 *cop-6* DNA; this cannot be explained by a denaturation hypothesis. (*iii*) More pE194 *cop-6*, but less pC223, single-stranded DNA was produced from *S. aureus* than from *B. subtilis*. (*iv*) Mutations in a hybrid plasmid that inactivate the replication region of pC194 but leave a second origin functional abolish the

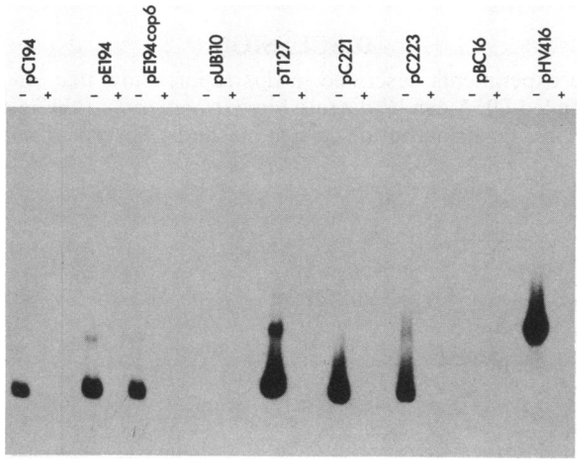

FIG. 5. Single-stranded plasmid DNA in *B. subtilis*. Lysates prepared from *B. subtilis* strains harboring the indicated plasmids were treated (+) or not treated (−) with nuclease S1 and run through an agarose gel. After transfer of DNA to nitrocellulose, filters were hybridized to the appropriate nick-translated probes, prepared from purified plasmid DNA.

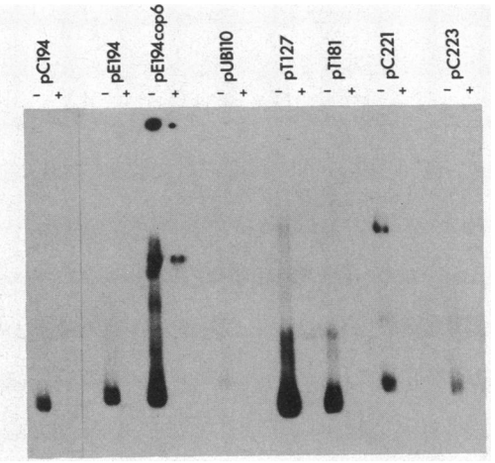

FIG. 6. Single-stranded plasmid DNA in *S. aureus*. Lysates prepared from *S. aureus* strains harboring the indicated plasmids were analyzed as described in the legend to Fig. 5. The specific activity of hybridization probes and the exposure time of autoradiographs were similar to those used for *B. subtilis* (Fig. 5).

capacity to produce single-stranded DNA (33). Therefore, we conclude that single-stranded DNA exists intracellularly and this may be a characteristic of many broad-host-range plasmids from Gram-positive bacteria. The study of plasmids from other organisms should allow a determination of how widespread that characteristic is.

We found that 20% of hybridization with a probe that contained both pC194 strands occurred at the position of the gel where single-stranded DNA would migrate (Fig. 1). Allowing for the fact that a double-stranded molecule gives a hybridization signal twice as intense as a single-stranded molecule, one-third of the plasmid copies were therefore single stranded. Since 15 double-stranded copies of pC194 exist per cell, 7–8 single-stranded copies must also be present. Similarly, for the majority of the plasmids studied here, the number of single-stranded DNA copies was not lower than one-third of the total number of plasmid copies. Notable exceptions are pUB110 and the related replicon pBC16, which generated very little single-stranded DNA.

The presence of relatively large amounts of single-stranded DNA raises two questions that tempt us to speculate. The first concerns the mechanism of single-stranded DNA formation, the second, its role.

Single-stranded DNA could be generated during plasmid replication, possibly by a rolling-circle-like mechanism as has been described previously for single-stranded DNA phages such as ϕX174, M13, f1, or fd (24, 25). Alternatively, single-stranded DNA could be generated by a D-loop replication mode, provided that the initiation of lagging-strand synthesis is delayed [as observed during replication of ColE1 in cell-free extracts lacking the DNA primase (26)]. A concatemer that would probably result could be subsequently resolved by a topoisomerase (27) into a double- and a single-stranded molecule. An entirely different way of generating single-stranded DNA, for which no examples are known to us, would be the degradation of one specific strand of the duplex molecule. Further work will be needed to distinguish among these alternatives.

The observation that single-stranded DNA is generated in different hosts by various plasmids may indicate that it has a role in the plasmid life cycle. What could that role be? We would like to discuss one of the several possibilities. It has been suggested that single-stranded DNA initiates recombination (28, 29) and shown that *B. subtilis* plasmids recombine very frequently (30). The role of the single-stranded DNA may be to stimulate intramolecular recombination and thus

prevent plasmid oligomerization, which has been shown to cause the loss of small, randomly partitioned plasmids such as ColE1 and CloDF13 from the host cell (31, 32). Experiments designed to modify the amounts of the single-stranded DNA generated by a plasmid may allow this hypothesis to be tested.

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