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The Family of Highly Interrelated Single-Stranded Deoxyribonucleic Acid Plasmids

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INTRODUCTION

Numerous plasmids of gram-positive bacteria have been identified, analyzed for their properties, and used as cloning vectors. The range of species from which plasmids have been isolated is broad, yet the properties of most of these plasmids indicate that a common gene pool had been used in their formation (36, 76). The regions of homology may involve essential or nonessential genes, and a single plasmid has composite character, comprising the antibiotic resistance gene of one plasmid, the replication region of another, etc. The relatedness of 14 of these plasmids is shown schematically in Fig. 1.

How did such a highly interrelated yet widespread family of replicative elements arise? A recent finding provides a clue; the plasmids under study, of which more than a dozen are already sequenced, all replicate via a single-stranded deoxyribonucleic acid (ssDNA) intermediate, probably by rolling-circle replication (RCR) (82, 83). This particular group of plasmids is referred to here as ssDNA plasmids. The production of ssDNA and the RCR mechanism have significant consequences for the recombination capacity of these plasmids; both homologous and illegitimate recombination are greatly stimulated, compared with frequencies observed in the chromosome. The high recombination capacity of these plasmids may accelerate their dissemination and explain why they are so interrelated. Discrete classes of recombination events are shown to occur as a direct consequence of RCR, i.e., homologous recombination between long homologous repeats (61); illegitimate recombination between short direct repeats (7, 38, 69; L. Jannière and S. Ehrlich, submitted for publication); formation of linear multimeric species of plasmids carrying certain DNA insertions in a wild-type background (23); recombination arising from aberrant replicative initiation or premature termination (22, 53); and, in some plasmids, a site-specific recombination system for which the effects of plasmid replication were not analyzed (18).

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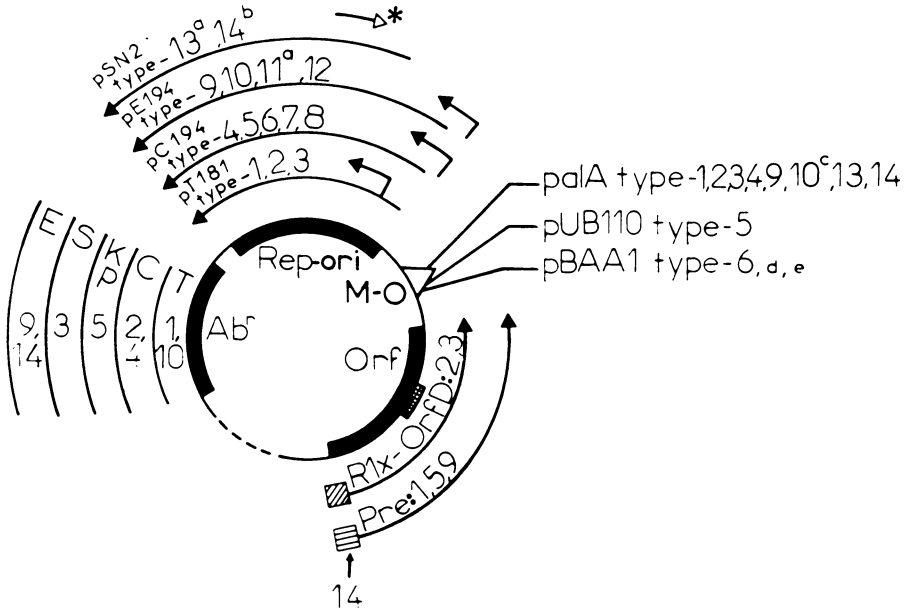

FIG. 1. Regions of relatedness among 14 ssDNA plasmids. A prototype ssDNA plasmid is shown in the inner circle, showing four functions commonly present. Plasmids listed together on the outer lines or arrows have homology in the corresponding function, as deduced by DNA and/or amino acid comparison. Plasmids are as follows: 1, pT181; 2, pC221; 3, pS194; 4, pC194; 5, pUB110; 6, pBAA1; 7, pCB101; 8, pIJ101; 9, pE194; 10, pMK158; 11, pADB201; 12, pSH71; 13, pSN2; 14, pIM13 (for further details, see Table 1). Abbreviations and symbols: Rep, replication protein required for plus origin (**ori**) activity; ⟵, origin, either embedded or upstream of *rep*, and direction of replication; ⟶ *, uncharacterized plus origin for which the direction of replication is deduced by the active orientation of the minus origin *palA*; Ab[r], antibiotic resistance (T, tetracycline; C, chloramphenicol; K, kanamycin, P, phleomycin; S, streptomycin; E, erythromycin); Orf, open reading frame(s), encoding either Pre (protein for recombination [18]) and its target site, RS_A, just upstream (▤) (18), or R1x and OrfD, overlapping polypeptides involved in the formation of a relaxation complex at a site just upstream (□) (76); ▦, *comp*, a locus analyzed only for pT181 (19, 20, 35) and pUB110 (Polak, personal communication). The order of functions on this circular representation is not fixed. ----- on the primary plasmid outline represents unrelated or uncharacterized sequences which may be present. [a]pSN2 and pADB201 have been fully sequenced, and each contains only one open reading frame larger than 60 amino acids, presumed or inferred to be involved in replication; [b]a plasmid identical to pIM13, pNE131, has been isolated from *S. epidermidis* (48, 75); [c]the M-O of pMK158, a streptococcal plasmid, has been described as *palA*-like (12), but the *palA* M-O (24) from a staphylococcal plasmid is nonfunctional in *Streptococcus* spp. (12); [d,e]the M-O of plasmid pBAA1 (14) is also present on *Bacillus* plasmids pLS11 (10) and pTA1060 (5).

The ssDNA plasmids represent an important family of replicons. The purpose of this article is to simplify future plasmid analyses by describing their characteristics, with emphasis on the unifying features. Methods of identifying new members of this family are given. Classification of plasmids according to their mode of replication should prove useful in the construction of cloning vectors. It should be noted that little is known of plasmids that are isolated from gram-positive bacteria and that are not of the ssDNA type. However, certain plasmids which do not share any of the common features identified for ssDNA plasmids probably replicate differently. For those that have been tested, e.g., plasmids pAMβ1 and pTB53 (Jannière and Ehrlich, submitted), differences in behavior were noted.

Several recent reviews may be of interest, since they emphasize different characteristics of the ssDNA replicons. References 2 and 58 discuss the replication of ssDNA bacteriophages; references 63, 66, and 76 and R. Novick, Annu. Rev. Microbiol., in press, discuss the organization and function of ssDNA plasmids of *Staphylococcus aureus*; and reference 17 discusses recombination in ssDNA plasmids.

PLASMID REPLICATION

Replication Mechanism for the Normal Case

For two plasmids, pT181 and pC194, detailed studies have demonstrated that replication occurs by RCR (22, 40, 45). The high degree of homology of the Rep proteins and plus origins of these two plasmids with those of at least seven other sequenced plasmids strongly suggests that all these plasmids replicate in a similar way, i.e., by RCR (22, 76). The RCR mechanism has been extensively studied for ssDNA *Escherichia coli* bacteriophages (see reference 2 for a review on mechanism).

Three plasmid-encoded elements are used for RCR: a plus origin, a replication protein (Rep), and a minus origin (M-O). The replication mechanism is shown schematically in Fig. 2. Rep (shown for pT181 to have topoisomerase [i.e., nicking-closing] activity [45]) recognizes an origin sequence and produces a nick to initiate replication (step 1). This event triggers displacement of the plus strand and polymerization of a new plus strand by 3′-OH extension from the nick (step 2). Then Rep recognizes a termination sequence, which overlaps the origin sequence, and produces a second nick to generate one fully replicated strand and an ssDNA monomer of the displaced strand (step 3). Finally, Rep ligates the ends of the ssDNA to form a circle, which is detectable as a free molecule (step 4). The nicks that initiate and terminate a round of plus-strand synthesis occur at the same site (2, 22). The M-O serves as an efficient initiation site, recognized by host factors, for the conversion of circular plus-strand ssDNA to double-stranded DNA (dsDNA) (step 5). The formation of the dsDNA plasmid product (step 6) marks the completion of one productive cycle of replication, in which two plasmids are generated from a single one. If the plus

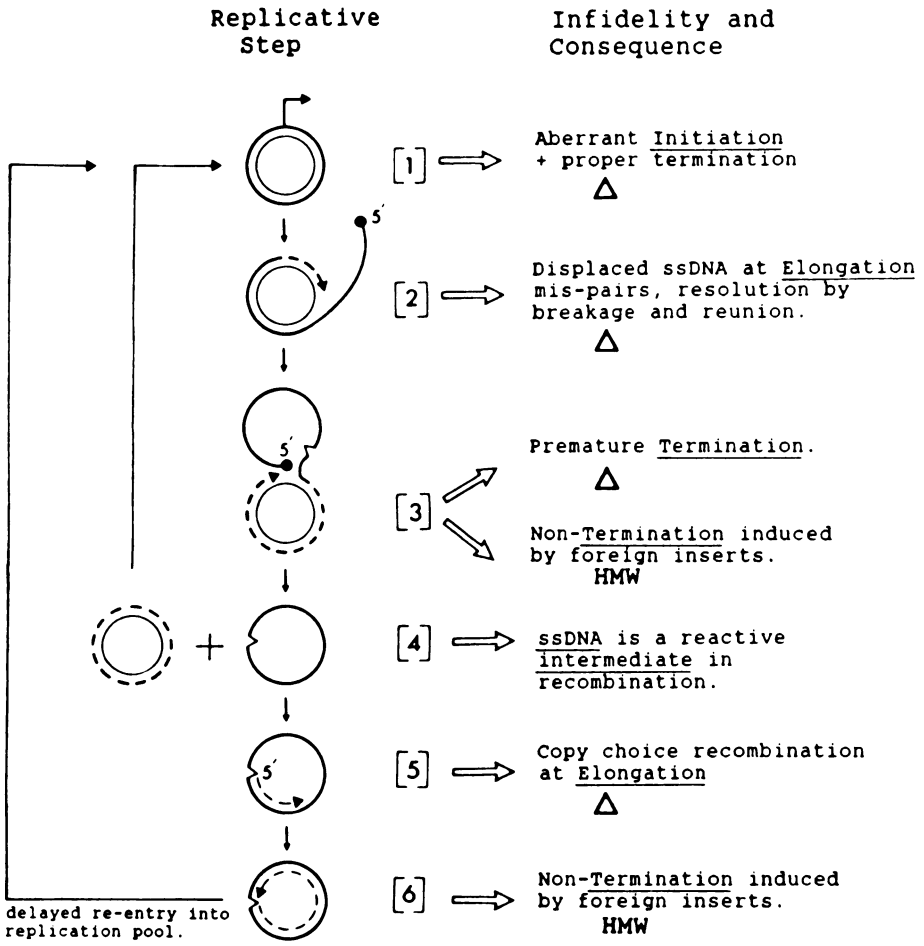

FIG. 2. Stages of normal RCR, with indications of places where nonstringency of specific steps may provoke rearrangements. Symbols: ▬, ▪ ▪ ▪, plus strand; ——, – – –, minus strand; ▬, ——, parental DNA; ▪ ▪ ▪ ▪, – – – –, newly synthesized DNA; ter/ori, terminus-plus origin overlapping region; ↱, direction of replication; <, secondary structure at the M-O. (See the text for a description of the steps.) Note that the molecule using the minus strand as the template is the first to reenter the replication pool. To the right of each step in the replication cycle are listed the imprecisions in the replicative steps which may result in a recombination event. Δ, Deletion formation.

strand is not converted to dsDNA, replication will be nonproductive and will result in the synthesis of one dsDNA plasmid and the accumulation of ssDNA.

Plus origins. Numerous plus origins have been analyzed. They are localized either upstream of, or within, the Rep open reading frame (Fig. 1). The fully active plus origins of pC194 and pT181 have been localized to 55 and 70 base pairs (bp), respectively (17a, 22). As mentioned above, the plus origin is recognized twice by Rep in the replication cycle, first for initiation and again, after the plus strand is displaced and a new strand is synthesized, for termination. The initiation and termination recognition sites of analyzed origins are overlapping but nonidentical, as demonstrated for the analogous class of *E. coli* ssDNA phages (2), as well as for ssDNA plasmid pC194 (22). Most origins, with the exception of the pUB110 origin, contain sequences having a potential for secondary hairpin structures. It is not known whether such structures play a role in replication and, if so, whether they are recognized at the initiation or termination step. The plus origins thus far identified are grouped into three types, according to homologies with other plus origins. The strongest homologies within the plus origins (Fig. 3) appear in the regions surrounding the nick sites (indicated in Fig. 3). Consensus sequences were also found between *E. coli* ssDNA phage origins and certain ssDNA plasmid origins (22).

All pairs of plasmids having homologous plus origins also have corresponding homologies (sometimes less stringent) in their Rep proteins. Furthermore, the amino acid motif around the active site of the ϕX174 Rep protein (84) is conserved in the Rep proteins of *S. aureus* plasmids pC194, pUB110 (22), and other analyzed plasmids sharing the consensus origin sequence (Fig. 4), suggesting that the mechanism of nicking at the active site between these plasmids and ϕX174 is analogous. A search of the published sequence (39) of *Streptomyces lividans* ssDNA plasmid pIJ101 (39, 71; J. Pigac, V. Gamulin, D. Vajaklija, Z. Toman, and H. Schrempf, Abstr. 5th Int. Symp. Genet. Ind. Microorg., p. 41, 1986) revealed a similar amino acid motif in the Rep protein (Fig. 4). The *Streptomyces* ssDNA plasmid is 72% G+C rich (as opposed to 30 to 40% G+C rich for the others). This may explain why the consensus origin sequence was not found, although a sequence bearing structural but not strict sequence similarity to the pC194 origin is present upstream of the Rep open reading frame (bp 1327 to 1269 of the published pIJ101 sequence [39]). It is not known whether this sequence has origin activity.

M-Os. The M-Os of several ssDNA plasmids have been

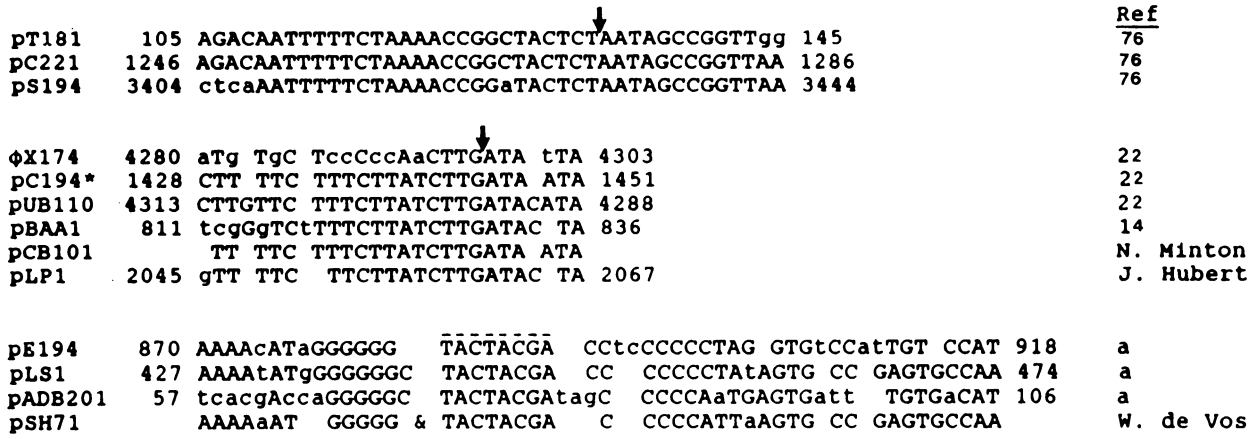

FIG. 3. Consensus sequences (left to right, 5′ to 3′) within the plus origins of ssDNA plasmids. Only the stretches of origin sequences showing strong homologies are presented. Nucleotides in capital letters are homologous; those in lowercase letters are nonhomologous. Nucleotide positions of sequences are given according to published maps. Vertical arrows and hyphenated line indicate replication nick sites and localization, respectively. Sequences are lined up to optimize homologies. In all cases of origin homologies, there are corresponding Rep protein homologies. Symbols: *, sequence numbering according to Dagert et al. (11); a; data compiled for origins related to pE194 (Sozhamannan et al., in preparation), pE194 (29), pLS1 (47), and pADB201 (4); &, there is a 23-bp intervening sequence aaaggaagcgaattt tgcttccg, not present in the other origins.

analyzed (4a, 12, 14, 24). The minimal sequences in all cases are large (at least 130 to 220 bp) and contain imperfect palindromic structures. All M-Os show orientation-dependent activity. DNA sequences of three unrelated M-Os are presented in Fig. 5, in which palindromes are indicated. Conversion is mediated by host-encoded factors (4a, 24). Replication is initiated in each of the three types of M-Os in vivo by host-encoded ribonucleic acid (RNA) polymerase, since rifampin blocks conversion (4a; L. Boe and A. Gruss, unpublished data). RNA polymerase is known to initiate replication at the M-Os of the filamentous ssDNA phages (2).

The properties of an ssDNA plasmid in different hosts may depend on whether the M-O is active. Many of the M-Os are host specific (4a, 12, 24); only the M-O of pUB110 is known to function in more than one host (4a). In all hosts, a plasmid lacking an active M-O is still viable, but accumulates ssDNA; conversion in these cases initiates only nonspecifically, at a reduced frequency (4a, 24, 77). In some hosts, such as *S. aureus* (24), *Streptococcus pneumoniae* (12), and *Streptomyces lividans* (13), deletion of the M-O also causes decreased plasmid copy number and, in the first two cases, pronounced plasmid segregational instability. In contrast, the M-O does not affect plasmid copy number in *Bacillus subtilis* (4a, 14, 24).

Copy Number Control and Its Role in Segregational Stability

Maintaining an upper limit to plasmid copy number may be necessary to ensure the viability of the host. In addition, to prevent plasmid loss, copy number controls must also respond rapidly in a cell inheriting few copies. This is particularly important in the absence of a separate partitioning locus (*par*). No evidence exists for a *par* locus on the ssDNA plasmids, in the context of its original definition, i.e., membrane attachment sites that physically aid segregation of sufficient numbers of plasmids such that they become established in each daughter cell (37). In plasmid pT181, the only mutations that seem to affect plasmid stability are those that affect replication functions (19, 20, 24, 35; A. Gruss and R. Novick, unpublished data) (see below). The *par* function, which is reported to reside on plasmid pLS11 (10) and which is also found in plasmids pTA1060 (5) and pBAA1 (14), was later revealed (on pBAA1) to be the M-O (14). In ssDNA plasmids, stability seems to be coupled with replication and not with a discrete *par*-like function.

Known copy number control elements affect the initiation step. Other modes of copy number control may act at other steps, e.g., elongation or conversion of ssDNA to dsDNA.

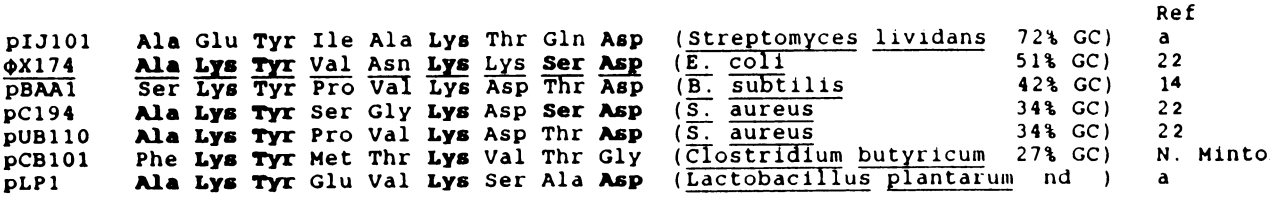

FIG. 4. Rep proteins of pC194-like origins have a conserved region related to the enzymatic active site of ΦX174 Rep protein (underlined) (84). The Percent G+C contents of the hosts are listed to indicate distance between the species. Corresponding DNA plus origin homologies are shown for these plasmids in Fig. 3, with the exception of pIJ101, the ssDNA plasmid (71; Pigac et al., abstract), isolated from *Streptomyces lividans*. On the level of plus origin homology, structural but not strict sequence similarity was found between the pC194 origin (22) and bp 1327 to 1269 of the published pIJ101 sequence (39), just upstream of the *rep* gene (A. Gruss, unpublished observation). It is not known whether this sequence has origin activity. a, Homologies found in pIJ101 and pLP1 are based on observation of DNA sequences; nd, not determined.

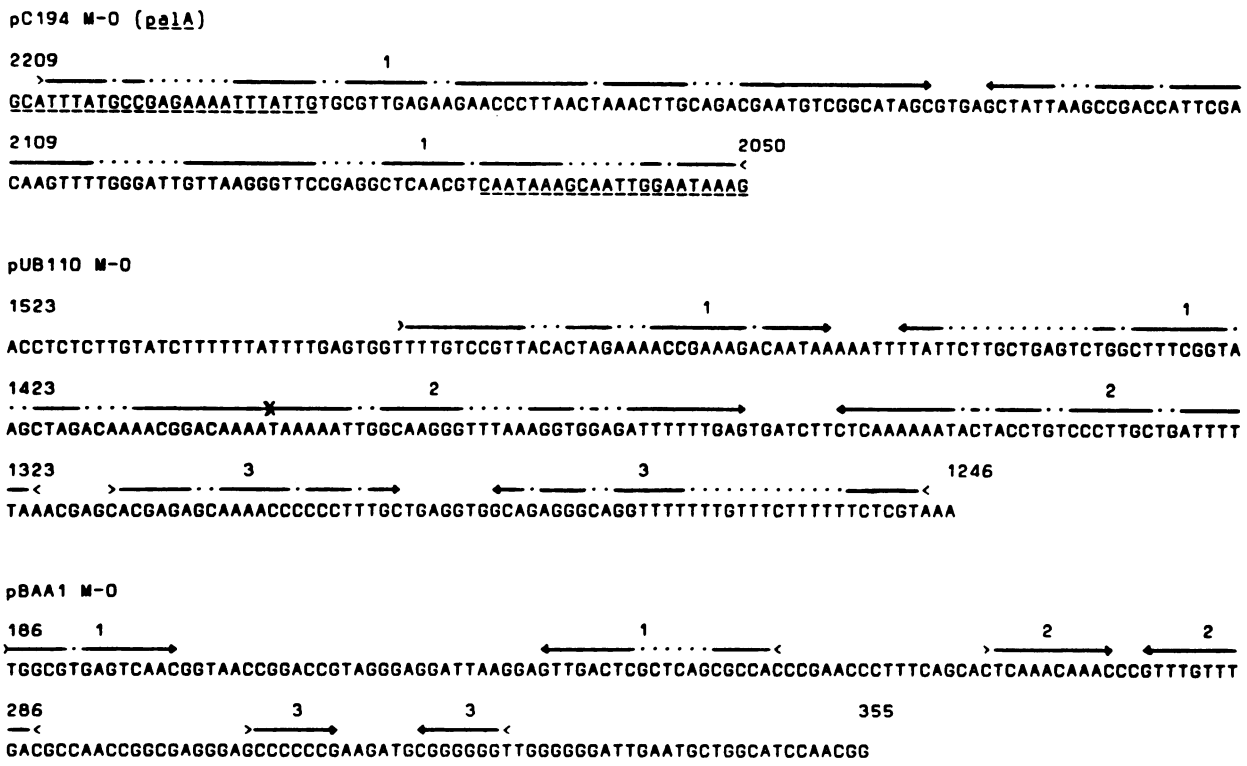

FIG. 5. DNA sequences of three M-Os. Palindromes are indicated by arrows above the DNA sequences; dots show positions of nonpalindromic bases. Arrows with the same numbers above form a single palindrome. Variants of the first M-O, *palA*, have been found on numerous staphylococcal plasmids. The M-O *palA* is functional in *S. aureus*, but not in *B. subtilis* (24). The second is the M-O of pUB110 and is the only one thus far that is known to function in at least two hosts, *S. aureus* and *B. subtilis* (4a). The third has been found on *Bacillus* plasmids pBAA1 (14), pLS11 (10), and pTA1060 (5).

The rate of elongation can be strongly affected by the DNA sequence and consequently can affect the plasmid copy number; this was demonstrated by observing the effects of insertion of a 22-bp termination sequence into pUC plasmids (27). Similarly, the efficiency of initiation of conversion of ssDNA to dsDNA can determine the plasmid copy number (Fig. 2). It remains to be shown that these steps can be manipulated for copy control.

Most of the data presented below on copy control at the level of initiation derive from plasmid pT181. The information is presented here with the speculation that analogous systems of copy control and stability are operational on other ssDNA plasmids.

Regulation of Rep synthesis. Plasmid copy number control by modulation of Rep expression has been extensively studied for pT181 (see reference 67 for a review). Regulation is achieved by the production of countertranscript (CT) RNA, which modulates either transcription or translation of the *rep* messenger RNA (mRNA), or both, and keeps the copy number within a limited range. Certain point mutations affecting the CT RNA have increased copy numbers (9, 64). Mutants in which the CT RNA is not synthesized have a much increased copy number (50-fold [9, 64]), but are not lethal. This suggests that other factors prevent runaway replication. Some of these are as follows: (i) certain high-copy-number plasmids are accompanied by deletion derivatives, which may bind to and thus deplete Rep (34); (ii) host factors (possibly rate limiting) besides Rep regulate the plasmid copy number and might prevent runaway replication (34); (iii) if ssDNA is accumulated at high concentrations, it could interfere with replication by titrating Rep (Rep binds to ssDNA [44]); (iv) Rep, as shown for *E. coli* plasmid R6K (79), may be less active at high concentrations; (v) the origin may be refractive to overreplication (see below).

When a daughter cell inherits too few copies of a plasmid, the imbalance is rapidly adjusted by overreplication (28, 63). This is expected if the CT RNA (which is present in large quantities [67] and would therefore be equipartitioned) decays rapidly. In this case, it would not inhibit plasmid replication in daughter cells (see below). Overreplication of plasmids in cells receiving too few copies is a sensitive control that responds to rapid fluctuations in copy number.

Availability of origin as a mechanism of copy number control. Several factors affect the efficiency with which an origin is recognized. Increased transcription through the pBR322 origin in the direction opposite to that of replication (81) reduces replication efficiency, whereas transcription through *oriC* enhances replication (3). Plasmid superhelicity affects the efficiency of replication initiation in pBR322 (55). Furthermore, hemimethylated pBR322 (the product of a round of replication) is not recognized as a substrate for replication (78). Therefore, if the necessary effectors are present but the origin is not readily available, initiation may be inefficient. Such factors may have a large effect on plasmid copy number and hence on copy control.

A novel locus, *comp*, affecting the efficiency of origin utilization, has recently been identified on plasmid pT181 (19, 20, 35). A *comp* mutant plasmid in *S. aureus* is unable to compete successfully for Rep in the presence of a *comp*$^+$ plasmid and requires a longer period than the wild-type plasmid to repopulate a cell if its copy number is reduced and then derepressed (20). *comp* is thought to facilitate Rep

recognition of the origin, possibly by affecting the superhelicity of origin sequences (19). An analogous locus was also identified in pUB110 (72; J. Polak, unpublished data). Normally, pT181 *comp* is located about 1,200 bp from the origin; its activity is orientation independent and is inversely proportional to its distance from the origin (20).

How can copy number control affect plasmid segregational stability? A simple proposal by Novick and co-workers presents an alternative to an independent partitioning system (20, 28, 63): plasmids, if partitioned randomly, should be present at random copy numbers in daughter cells after cell division. If a plasmid has a tight copy control system (e.g., high turnover rate of CT RNA), cells receiving fewer plasmid copies will rapidly adjust this situation by synthesizing Rep protein and undergoing compensatory overreplication (28, 63). However, if Rep protein is synthesized too slowly (e.g., owing to transcriptional repression of *repC* mRNA by an overactive CT RNA) or if the origin is not readily available for replication (e.g., owing to a *comp* defect), this adjustment may not occur fast enough to replete the plasmid copy pool. The copy number will remain low in these cells, and plasmids may be lost in subsequent cell divisions.

Since the activity of *comp* is inversely proportional to its distance from the origin (20), it follows that cloning of foreign DNA segments into an ssDNA plasmid diminishes the interaction between *comp* and the origin and hence lowers the ability of the plasmid to adjust its copy number upon cell division (20). Failure to rapidly correct copy number fluctuation may account for the reported segregational instability of these plasmids when they are used as cloning vectors (6).

INFIDELITY OF RCR

The replication of ssDNA plasmids does not always follow the normal system schematized in Fig. 2. Nearly every step in the process is either known or proposed to digress from its usual function, thus effecting rearrangements. In addition, RCR generates ssDNA; in every recombination process, ssDNA is a reactive intermediate. The rearrangements and the possible steps at which they occur are described below. Numbering follows the replication steps schematized in Fig. 2.

Step 1: initiation. Initiation at a pseudo-plus origin, followed by termination at the correct plus origin, provides a consistent explanation for the endpoints found in a deletion induced by pC194 replication (53).

Step 2: elongation and displacement during plus-strand synthesis. Elongation during plus-strand synthesis with concomitant displacement of plus-strand ssDNA may lead to initiation of homologous recombination by the displaced strand (52). Deletion frequencies between long direct repeats carried by ssDNA plasmids are about 1,000-fold greater than deletion frequencies of equivalent constructions in the chromosome (61). Insertion of an ssDNA plasmid in the *B. subtilis* chromosome also stimulates deletion and amplification of adjacent repeat sequences (62; M.-A. Petit, J. Mesas-Mesas, P. Noirot, and S. Ehrlich, submitted for publication).

Step 3: termination of plus-strand synthesis. Termination of plus-strand synthesis by a wobbly recognition of the termination sequence results in the formation of recombinants. Either the wrong sequence is recognized, which provokes precocious termination (step 3A), or the correct termination sequence is ignored (step 3B).

In step 3A, precocious termination of replication in ssDNA plasmids, recombinants are generated. In certain *E. coli* plasmid constructs comprising the ssDNA filamentous phage, pC194, and pBR322 sequences, the nick site at the phage replication origin is a deletion hot spot in *E. coli* (54). Likewise, in certain of these constructs, a deletion hot spot also exists within the pC194 sequence (53). It was hypothesized that this hot spot was the replication nick site (53), and it was later confirmed directly that this hot spot was in the pC194 origin region within an 18-bp sequence containing the nick (22). The other deletion endpoints were at originlike sequences (53, 54). The simple explanation for the formation of these deletions is the correct initiation at the origin and aberrant termination at sequences resembling the termination-origin sequence (22; M.-F. Gros, H. te Riele, and S. D. Ehrlich, submitted for publication).

The 18-bp sequence containing the pC194 nick site is also present in pUB110 (22). A cointegrate between pC194 and pUB110 replicons was previously reported to decombine at high frequencies at precisely this sequence (26). Studies by Gros et al. showed that the cointegrate is actually resolved by initiation at one origin and accurate and efficient termination at the 18-bp sequence of the second origin (22) (i.e., initiation at the pC194 origin and termination in the pUB110 18-bp sequence, and vice versa). Although 55 bp is required for Rep to recognize the pC194 origin, only 18 bp is required for Rep to mediate normal termination (22). Likewise, although pUB110 requires a larger origin for initiation, only 18 bp is necessary for normal termination (4a).The termination reaction can be degenerate, and so to study the termination process, a plasmid was constructed comprising the entire pC194 origin sequence and an insertion of the 18-bp termination sequence. In this plasmid, termination occurred prematurely at the 18-bp sequence. It was shown that certain base changes within the 18-bp sequence still provoked termination, albeit at reduced frequencies; thus, errors by the Rep protein in termination resulted in deletions (Gros et al., submitted).

In step 3B, the correct termination sequence is ignored. The presence of certain insertions of foreign DNA in any position within the ssDNA plasmids results in the formation of high-molecular-weight tandem multimers (HMW) (23). HMW are produced in the wild-type host with any ssDNA plasmid vectors, but not with a vector apparently outside this family (plasmid pAMβ1 was tested). It is hypothesized that the presence of a foreign DNA insertion interferes with normal termination of plus-strand replication. Notably, all shuttle vectors tested consisting of an ssDNA plasmid and pBR322 sequences produce HMW in gram-positive hosts; for these constructs, more than 70% of the plasmid DNA is present as HMW.

Step 4: release of circular ssDNA. Released circular ssDNA may be a reactive recombination intermediate, with possible physiological effects such as SOS induction (H. te Riele, S. D. Ehrlich, and R. D'Ari, unpublished data). Results of preliminary experiments suggest that ssDNA stimulates intermolecular homologous recombination (V. Vagner, personal communication).

Step 5: elongation during minus-strand synthesis. Elongation during minus-strand synthesis is subject to slipped mispairing recombination. Recombination, tested by using short (9-bp) direct repeats flanking an inactive transposon (i.e., inverted repeat sequences), is stimulated 150- to 1,500-fold in plasmids that replicate via an ssDNA intermediate compared with the chromosome or plasmids which do not generate ssDNA (38; Jannière and Ehrlich, submitted). A study of different-length direct repeats flanking inverted repeats in ssDNA plasmids in *B. subtilis* showed that dele-

tion frequencies are proportional to the length of the repeats (69). A copy choice recombination mechanism has been proposed (see reference 17 for a discussion), and results of a model system developed with *E. coli* give good evidence of this mechanism (7).

A difference should be noted, however, between *B. subtilis* and *E. coli* recombinations that are or are not stimulated by ssDNA. In *E. coli*, deletions between short repeats can occur at about the same frequencies as deletions between long repeats (D. Brunier, personal communication). In *B. subtilis*, deletion frequencies of short direct repeats are 10^5 to 10^6 times lower than deletion frequencies of long direct repeats (38). This implies that the copy choice mechanism of recombination, which is active in the deletion of short direct repeats (7), may be less frequent or better monitored in *B. subtilis* than the equivalent mechanism in *E. coli*.

Step 6: termination of minus-strand synthesis. The formation of HMW (step 3B) was also proposed to occur by a failure to properly terminate minus-strand synthesis. If the 3′-OH end is displaced, a nonterminating RCR would continue to form HMW.

PLASMID PROPAGATION AND HOST RANGE

The ssDNA plasmids are found in a large number of species, suggesting the availability of an effective means of transfer and a wide adaptability. Their transferability may be enhanced by their capacity to recombine in various ways during replication.

Plasmid Propagation

Plasmid transfer is known to occur by transduction, conjugation, and transformation. Both the mode of replication and specific functions on the plasmid may stimulate transfer.

Transduction. A thermosensitive replicon was used to show that in *S. aureus*, pT181 replication is required for the formation of packaged plasmid multimers (65). Also, a 5- to 10-fold-higher transduction frequency was observed for plasmids that accumulate ssDNA (M. Gennaro, personal communication). The involvement of replication and ssDNA in transduction may be explained if the ssDNA generated during normal replication serves as the template for rolling-circle multimers which are packaged in transducing particles. In seeming contradiction, it was reported that plasmid replication does not alter the transduction efficiency in *B. subtilis* (1). However, the thermosensitive replicon used in the latter study, pE194, accumulates ssDNA, which persists for several hours after plasmid replication is blocked at high temperatures (S. Projan, H. te Riele, and A. Gruss, unpublished observations). The high transduction frequency observed after a 2-h block of plasmid replication could be due to the persistence of ssDNA replicative intermediates during this period.

Conjugation. The studies of ssDNA plasmid transfer by conjugation are as yet preliminary. Several ssDNA plasmids can be mobilized in the presence of a conjugative plasmid in either *Staphylococcus* (50, 60) or *Bacillus* (43) spp. Mobilization (or relaxation) sites have been described for the staphylococcal plasmids pC221 and pS194 (74, 76). These sites are thought to be initiation sites for strand transfer. Mobilization proteins are also mapped on the same plasmids (74, 76). However, pUB110, which has no homologies in the relevant region, can be mobilized between various *Bacillus* spp. (43); thus, other as yet unidentified sites may mediate plasmid transfer in different hosts.

TABLE 1. ssDNA plasmids and their hosts of origin

ssDNA plasmid[a]	No. in Fig. 1	Host of origin	Reference or source
pT181	1	*S. aureus*	42, 76
pC221	2	*S. aureus*	73, 76
pS194	3	*S. aureus*	74, 76
pC194	4	*S. aureus*	22, 30
pUB110	5	*S. aureus*	46, 51
pBAA1[b]	6	*B. subtilis*	14
pCB101[b]	7	*C. butyricum*	57
pBC16		*B. cereus*	70, 72
pLP1		*L. plantarum*	J. Hubert
pIJ101[c]	8	*S. lividans*	39
ϕX174[d]		*E. coli*	2
pE194	9	*S. aureus*	29
pMK158[e]	10	*S. agalactiae*	8, 47
pADB201[f]	11	*M. mycoides*	4
pSH71	12	*Lactococcus lactis*	15
pSN2	13	*S. aureus*	41
pIM13	14	*B. subtilis*	75
pNE131[g]		*S. epidermidis*	48
pGRB[f]		*Halobacterium* sp.	80

[a] Plasmids are grouped according to homologies in plus origin replication functions. Other types of interrelatedness among several of these plasmids are shown in Fig. 1.

[b] Only the replication regions of these plasmids have been fully analyzed.

[c] pIJ101 generates ssDNA (71; Pigac et al., abstract). It is tentatively placed with the other plasmids in this group on the basis of amino acid homology of its Rep protein in the region thought to correspond to the enzymatic active site.

[d] Bacteriophage ϕX174 has DNA homology with the origins and encodes a Rep protein which shares significant homology around its enzymatic active site with the Rep proteins of the related ssDNA plasmids.

[e] pMK158 is the parent of pLS1, a well-characterized deletion derivative missing 1.1 kilobase pairs (8, 47).

[f] Native hosts of these plasmids are not gram-positive bacteria.

[g] pNE131 is nearly identical to pIM13 (75).

The formation of cointegrates between an ssDNA plasmid and a readily transferable plasmid may also facilitate propagation. Cointegrates mediated at either homologous or specific (nonhomologous) sites have been reported (see below).

Possible role of HMW in plasmid transfer. The role of HMW in plasmid transfer has not been analyzed. However, HMW DNA is, predictably, very efficient in competent cell transformation in *B. subtilis* (Gruss, unpublished data). One could speculate that owing to the predominantly linear multimeric structure of HMW (see above), it could readily serve as a substrate for all processes of DNA transfer.

Host Range

A list of ssDNA plasmids and their hosts of origin is given in Table 1. Plasmids that have pE194-like origins and that show homology in their plus origins and Rep proteins are found in hosts as distant as *S. aureus* and *Mycoplasma mycoides* (4) (Fig. 1 and 3). Staphylococcal plasmid pC194 (33) has been established in *B. subtilis* (16), *E. coli* (21), and *Streptococcus pneumoniae* (12); in contrast, staphylococcal plasmid pUB110 (46), which is closely related to pC194 in the replication region (22), does not replicate in *E. coli* (H. te Riele and A. Gruss, unpublished data), although it does replicate in *B. subtilis* (25) and *Clostridium acetobutyricum* (49).

The plus origin, Rep protein activity, and M-O recognition are involved in successful host adaptation. Although M-O recognition is not strictly required, a plasmid lacking an active M-O may exhibit very different characteristics (see above).

The host range of an ssDNA plasmid may be broadened by cointegrate formation. For example, *B. cereus* plasmid pBC16 has been isolated from *Streptococcus faecalis* (70) as a cointegrate (mediated by a 380-bp region of homology) with a second plasmid which is replicative in this host; however, it has not been found autonomously (70). By piggybacking, pBC16 seems to have extended its host range. If the cointegrate plasmid is introduced by DNA transformation into *B. subtilis*, pBC16 spontaneously recombines to form an autonomous replicon (the second replicon is nonviable) (70).

The reverse situation, in which an ssDNA plasmid broadens the host range of a second plasmid, has also been found. A pUB110-like plasmid has been found in cointegrate form with a second plasmid to form pTB19; in this case, the extended host range is offered by pUB110, which replicates in thermophilic *Bacillus* spp., whereas the other replicon does not (31, 32).

Certain cointegrates form at a specific rate, RS_A (Fig. 1), present on several ssDNA plasmids isolated from *S. aureus* (68). Formation is mediated by a plasmid-encoded function, *pre* (plasmid recombination) (18). Cointegrate formation by this mechanism, as well as resolution to monomers by a different mechanism (e.g., initiation-premature termination [see above]), may enhance plasmid diversification. Two schemes of cointegrate formation leading to diversification are shown in Fig. 6. Transient cointegrate formation may thus allow a DNA molecule a broader host range; resolution of the cointegrate could generate a plasmid better adapted to the new host.

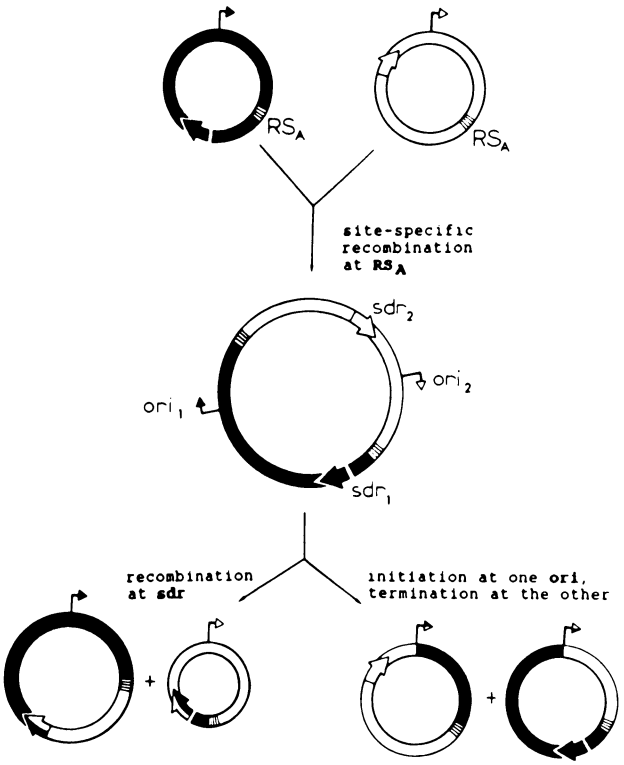

FIG. 6. Diversification scheme for ssDNA plasmids by cointegration via one pathway, and deletion formation by a second pathway. Cointegrate formation may occur at RS_A sequences by site-specific recombination (18, 68), as shown here, or at regions of homology. Recombination through short direct repeat (**sdr**) sequences arbitrarily present on the plasmid could give rise to novel recombinant plasmids (left). Initiation at one origin, **ori**, and premature termination at the heterologous origin (or another sequence resembling the origin) would also give rise to a hybrid plasmid (right) (22; Gros et al., in preparation).

DETECTION OF AN ssDNA PLASMID

Although little is known of the non-ssDNA-type plasmids, the numerous common properties of the ssDNA plasmids make it relatively simple to recognize whether a newly isolated plasmid is in this family. Described below are four means of identifying an ssDNA plasmid and two ways of analyzing aspects of plasmid replication.

Means of Identification

(i) Presence of sequence homologies. The presence of sequence homologies between the new plasmid and a known ssDNA plasmid, particularly within the replication region, offers a strong indication that the new one is of the ssDNA type. Of significance is conservation of sequences around the replication nick site at the origin. Figure 3 shows short sequence homologies between the origin regions of either pC194 (22), pT181 (76), or pE194 (57; S. Sozhamannan, P. Dabert, A. Gruss, and S. D. Ehrlich, manuscript in preparation) with other plasmids.

Similarly, amino acid homologies, although less stringent, may be found between the Rep proteins (Fig. 4). Since homology at the protein level allows more degeneracy of the DNA code, it may be easier to identify homologies at this level. Thus far, homologies based on either DNA or amino acid sequence have been found to include only the ssDNA plasmids.

(ii) Presence of plasmid ssDNA. The presence of plasmid ssDNA (as tested by the procedure of te Riele et al. [83]) identifies a new family member. If there is no detectable ssDNA, it may be because there is an efficient M-O. It may be worthwhile to (i) test for ssDNA in a foreign host, since many M-Os are inactive outside their native host; (ii) introduce deletions in the plasmid to delete M-O sequences, which are not required for replication (a good host for manipulation is *B. subtilis*, because the absence of an M-O does not interfere with plasmid copy number [24]); or (iii) add rifampin to a culture of the plasmid-containing strain, since the M-Os tested thus far are RNA polymerase dependent, and rifampin addition results in the accumulation within a short time (less than 1 h for *B. subtilis* or *S. aureus*), of ssDNA, which can be readily visualized on a gel by Southern blot hybridization (4a).

(iii) HMW production upon insertion of pBR322. Does the plasmid produce HMW upon insertion of pBR322? Since one of the first things done with newly isolated plasmids is to make shuttle vectors for transfer into *E. coli*, a construct which can be used to test for HMW is often already available. If a gram-positive strain containing a hybrid of the plasmid and pBR322 (or numerous other foreign insertions) produces HMW, the plasmid is likely to be of the ssDNA type (23).

(iv) Unstable inserts. If cloning attempts give rise to a high frequency of deletions, it is likely that the cloning vector is of the ssDNA type. Comparative studies of the stability of cloned inserts in an ssDNA-type plasmid and pAMβ1, a

plasmid that does not seem to replicate by RCR, show that random foreign inserts in the ssDNA plasmids are smaller and significantly less stable (Jannière and Ehrlich, submitted).

Analysis of Replication

If the plasmid is known to be of the ssDNA type, the following two ways of analyzing its replication may be used.

(i) Direction of replication of a plasmid. The activity of M-Os is orientation specific (4a, 24). By cloning a segment containing the M-O of the new plasmid onto a known plasmid lacking its own M-O, the active orientation of the M-O with respect to the origin will be determined. Alternatively, if the M-O sequence is homologous to a known M-O, the direction of replication can be deduced.

(ii) Mapping the plus origin by the initiation-termination reaction. The initiation-termination reaction can be used to map the replication origin. If homology exists between the new plasmid and the origin sequences of a known plasmid, the unknown nick site of replication can be mapped by generating a cointegrate between the two plasmids and mapping the deletions that occur in the orientation for which both plasmids replicate in the same direction. Initiation-termination is an efficient process (see above) (22). Thus, if initiation occurs at one origin and termination occurs at the homologous sequence of the other origin of the cointegrate, the nick site can be mapped to within a few nucleotides (4a; Sozhamannan et al., in preparation).

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