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Plasmid pSC101 Harbors a Recombination Site, *psi*, Which Is Able To Resolve Plasmid Multimers and To Substitute for the Analogous Chromosomal *Escherichia coli* Site *dif*

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Plasmid pSC101 harbors a 28-bp sequence which is homologous to *dif*, the target site of the XerC/XerD-dependent recombination system in *Escherichia coli*. Using a technique which allows very sensitive detection of plasmid loss, we show that recombination at this site, termed *psi* for pSC101 stabilized inheritance, causes a moderate increase in pSC101 stability. The role of the *psi* sequence in site-specific recombination has been explored in two other contexts. It was cloned in a derivative of plasmid p15A and inserted into the chromosome in place of *dif*. In the first situation, *psi* activity requires accessory sequences and results in multimer resolution; in the second situation, it suppresses the effects of the *dif* deletion and can promote intermolecular exchanges. Thus, *psi* is a site whose recombinational activity depends on the context, the first in the *cer*/*dif* family known to exhibit such flexibility.

The *Escherichia coli* chromosome encodes two closely related recombinases of the λ integrase family, XerC and XerD. XerC was identified as a factor controlling ColE1 multimer resolution at the *cer* site (9). More recently, a gene showing sequence similarity to *xerC* was found in the *recJ* region (17). This gene, *xerD*, is also involved in site-specific recombination, and all available evidence indicates that both XerC and XerD functions are required to complete the exchanges between two target sites (6).

XerCD target sites have been found on several replicons: R1 (8), ColE1 (28), ColK (25), CloDF13 (13), pSC101 (14), phage cf16-v1 of *Xanthomonas campestris* (8, 10), and the chromosomes of *E. coli* (5, 8, 14) and *X. campestris* (8, 10). These sites are 28 or 30 bp long and are organized as two well conserved segments of 11 bp flanking a 6- or 8-bp central region of variable sequence (Fig. 1). XerCD-controlled site-specific recombination follows two routes, depending on the pair of sites considered: either it is oriented towards multimer resolution, a situation first described for exchanges between ColE1 *cer* sites, or it operates intermolecularly as well as intramolecularly, a situation found for exchanges involving chromosomal *dif* sites.

The *cer* locus of the ColE1 plasmid is the archetype of sites displaying a strong bias towards intramolecular exchanges and multimer resolution (28). Its presence improves plasmid stability in *E. coli* by maximizing the number of segregation units at division (27, 28). Importantly, the core sequence alone is inactive, and the presence of accessory sequences and factors is required not only for directionality of exchanges but also for the overall recombinational activity of the site. The core 30-bp sequence (Fig. 1), which is recognized by XerCD (6) and contains the site of strand exchange (25, 26), is embedded in a longer sequence of 280 bp (29) which binds accessory factors required for full activity. These factors are ArgR, the arginine biosynthesis pathway repressor which binds an ArgR consensus binding site 80 bp distant from the crossover region (24), and PepA, a leucine aminopeptidase (23).

The first site known to be active in intermolecular recombination was a hybrid between *cer* of ColE1 and its homolog on CloDF13, *parD*. Actually, *cer-parD* hybrids work only towards resolution or are able to promote intermolecular exchanges depending on the size of the central part (6 or 8 bp), although their two arms share identical sequences, and it has been proposed that the distance between the two arms could be a factor for discriminating between the two classes (26). Indeed, the best known natural representative of a multidirectional site, *dif*, displays a 6-bp central region. It is located at 34 min in the replication termination region of the *E. coli* chromosome (8, 14) and shows an extensive homology with the *cer* core sequence (Fig. 1). Strains in which *dif* has been deleted display a filamentation phenotype in which only a fraction of the population form filaments (14) (see Fig. 8). This phenotype could be caused by abortive partitioning of chromosome dimers. The role of *dif* is supposed to be the monomerization of chromosome dimers formed by sister chromatid exchange during replication (5, 8, 14). Exchanges between a plasmid-borne *dif* site and the chromosomal one are frequent enough to allow high maintenance of the free form of a replication temperature-sensitive plasmid at high temperatures (8). Recombination at *dif* does not require ArgR and PepA (5).

Thus, in the XerCD recombination system, a pair of identical core sequences may behave in three different ways, depending not only on the core sequence but also on its genetic context: (i) no exchange is catalyzed (we call such a pair inactive), (ii) the exchange works in resolution only and requires accessory sequences (the pair is termed constrained), and (iii) the exchange reaction is relaxed and can form fusions (or inversions) as well as carry out resolution, and since this behavior does not require accessory sequences, the pair is termed unconstrained. A pair of *cer* sites can be either inactive or constrained, whereas *dif* sites have always been found to be unconstrained. We report here our analysis of the behavior of the *cer*/*dif* homolog present in plasmid pSC101 (14), which we call *psi* for pSC101 stabilized inheritance. Our results show that, unlike other sites of this type, a pair of *psi* sites may

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ColE1 *cer*	GGTGCGTACAATTAAGGGATTATGGTAAAT
E.coli *dif*	GGTGCGCATAA TGTATA TTATGTTAAAT
pSC101 *psi*	GGTGCGCGCAA GATCCA TTATGTTAAAC
pif (*dif-psi* hybrid)	GGTGCGCGCAA GATCCA TTATGTTAAAT

FIG. 1. Sequences of target sites recognized by XerCD recombinases. The sequences are aligned on the two distal arms to emphasize their conservation and the variability of central regions. Except for *pif* (this work), these sequences are redrawn from in reference 5 for *cer*, in references 8 and 14 for *dif*, and in reference 14 for *psi*. On *dif*, the left half-site is recognized by XerC and the right half-site is recognized by XerD (6). Note that the sequence of the hybrid site *pif* is nearly identical to that of *psi*, except for the rightmost nucleotide, which comes from *dif*. As drawn here, the left side of *pif* comes from pSC101, and its right side comes from the chromosome.

belong to any of the above classes, depending upon the genetic context in which the sites are inserted.

MATERIALS AND METHODS

Strains and plasmids. The strains used are listed in Table 1. All strains used in plasmid stability studies were derived from LN2666, a spontaneous streptomycin-resistant mutant of CB0129 (F^- W1485 *leu thyA deoB* [or *deoC*] *supE*) (3). The plasmids used and their constructions are presented in Table 1.

Media and general procedures. All in vivo experiments were carried out in rich Luria medium (22). Indicator medium for *lacZ* derepression was L agar plus 20 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) per ml (19). The antibiotic concentrations used were 200 μg of streptomycin per ml, 20 μg of chloramphenicol per ml, 50 μg of tetracycline per ml, 50 μg of ampicillin per ml, and 50 μg of spectinomycin per ml. All cloning, PCR amplification, and sequencing experiments involved standard procedures (22).

Multimer detection. Plasmid DNA was extracted from cultures grown overnight in Luria medium with selective pressure for plasmid presence. A standard alkaline lysis procedure (22) was followed, except for the addition of a protein precipitation step with 2.5 M (final concentration) ammonium acetate after the phenol-chloroform extraction step. Care was taken to keep the cell concentration below 10 units of optical density at 550 nm per ml at lysis to minimize contamination of plasmid DNA by chromosomal DNA during extraction. Plasmid DNA was electrophoresed on a 0.4% agarose gel for 3 h at 2.5 V/cm, stained with 1 μg of ethidium bromide per ml, and photographed.

Assays of plasmid stability and integration. Tests for plasmid stability are detailed in Results. Site-specific intermolecular recombination between sites borne by a replication temperature-sensitive plasmid (all derived from pSC101) and the chromosomal sites was detected by using an integration test and determining the frequency of bacteria forming colonies at 42°C in the presence of an antibiotic that killed the plasmidless bacteria (8, 14).

Precise substitution of chromosomal segments. Use of plasmids of the pFC13 plasmid family (Fig. 2 and Table 1) for substitution of chromosomal segments involved the following steps: (i) cloning of the chromosomal segment of interest; (ii) in vitro insertion of an antibiotic resistance gene at the required position within this segment (either with or without association with deletion of a chosen sequence); (iii) introduction of the resulting plasmid into an *rpsL* (streptomycin-resistant [Sm^r]) mutant, which renders the cells not only chloramphenicol resistant (Cm^r) but also streptomycin sensitive (Sm^s), because of the dominance of $rpsL^+$ (15); (iv) selection for plasmid integration by selecting Cm^r (and Sm^s) clones at 42°C; and (v) selection for plasmid excision by selecting Sm^r (which should also be Cm^s) clones at 42°C, with maintenance of the selective pressure for the resistance marker tagging the chromosomal segment. To construct LN2843 (LN2666 *lacI*::Ω-Ap^r), the $lacI^+$ gene was first cloned into plasmid pFC20 (Table 1) from plasmid pAPT2 (20), and then a *Bam*HI Ω-Ap^r (12) was inserted at the *Bcl*I site of *lacI*. The disrupted gene was finally substituted for the chromosomal $lacI^+$ locus as described above. Deletion of *dif* and its replacement by *pif* or *psi* were performed following the same general procedure; details are provided in Results.

RESULTS

Systems for plasmid stability measurements. Two host plasmid systems, in which the plasmid loss results in either a selectable or a visually detectable phenotype, were used to test plasmid stability.

(i) Selectable phenotype. Plasmid pFC13 (Fig. 2 and Table 1) is a pSC101-based plasmid containing a temperature-sensitive replication mutation in *repA*, a chloramphenicol acetyltransferase (*cat*) gene, and a 600-bp fragment carrying $rpsL^+$, the wild-type gene for *E. coli* ribosomal protein S12. An *E. coli* strain carrying the *rpsL* allele, recessive to $rpsL^+$ (15), is streptomycin resistant. Such a strain carrying pFC13 is therefore Sm^s, whereas plasmid-negative segregants become Sm^r again. This plasmid-host combination provides a convenient and extremely sensitive assay for plasmid loss.

(ii) Detectable phenotype. Plasmid pAPT110 (20) is a p15A-based plasmid carrying the overproducing allele of the *lac* operon repressor, $lacI^q$. This plasmid (Fig. 2 and Table 1) carries no known multimer resolution system. In the absence of a *lac* operon inducer, repressor-negative (*lacI*::Ap^r) pAPT110-carrying bacteria are phenotypically Lac^+ and form white colonies (or blue-sectored colonies when plasmid loss is frequent) on X-Gal medium. Plasmid loss results in derepression of β-galactosidase synthesis and the formation of blue colonies on X-Gal medium.

Role of *psi* site in pSC101 stability. In pSC101, an oligonucleotide copy number plasmid with an active partition system (for a review, see reference 18), the *cer*/*dif* homolog, *psi*, is located between positions 6783 and 6810 (4), downstream from the essential replication gene *repA* and just beyond an *orf* of unknown function, which we termed *orfX*. As shown in Fig. 1, the *psi* and *dif* sequences are very similar in the two 11-bp flanking elements, especially the right-hand one, but the intervening sequences (6 bp) are different. Deletions of *psi* and its surrounding region were constructed (plasmids pDJ1 and pIM6), and they resulted in reduction of stability by two orders of magnitude compared with that of the wild type, as measured by the appearance of streptomycin-resistant colonies (Fig. 3). This increased instability is probably due to the *psi* deletion since a similar effect on stability was also observed for the original pDJ2 plasmid in $XerC^-$ and $XerD^-$ hosts (data not shown). Furthermore, a frameshift mutation as well as a deletion mutation of *orfX* (plasmids pIM8 and pIM11) (Fig. 3) had a destabilizing effect. Either OrfX could be an accessory factor necessary for *psi* activity, or it could act independently in the maintenance of pSC101. The roles of *psi* and *orfX* in pSC101 stability have not been further explored. Their importance in pSC101 stability is relatively modest, and this made it technically difficult to pursue the analysis of their roles in this plasmid. To deal with a larger range of effects, we inserted *psi* into pAPT110, a p15A-derived plasmid randomly distributed

TABLE 1. Strains and plasmids

Strain or plasmid	Characteristic(s) or description	Construction, origin, or reference
Strains		
STL116	AB1157 *xerD*::Tn*10.9*	17
DS981	*xerC*::*kan*	6
DS954	*argR*::Tn*5*	24
DS957	*pepA*::Tn*5*	23
Hfr16.99	Hfr KL16 *relA1 gal recA1 srl*::Tn*10*	Our collection
CB0129	F^- W1485 *thy leu*	3
LN2666	CB0129 *rpsL2666*	Spontaneous Sm^r derivative
LN2772	LN2666 Δ*dif*::*tet*	This work
LN2799	LN2666 Δ*dif*::*tet*::*pif*	This work
LN2843	LN2666 *lacI*::*amp*	This work
LN2903	LN2843 *xerD*::Tn*10.9*	P1 transduction from STL116
LN2904	LN2843 *argR*::Tn*5*	P1 transduction from DS954
LN2905	LN2843 *pepA*::Tn*5*	P1 transduction from DS957
LN2948	LN2843 *xerC*::*kan*	P1 transduction from DS981
LN2949	LN2843 *recA1 srl*::Tn*10*	Cotransfer from Hfr16.99
LN2974	LN2666 Δ*dif*::*tet*::*psi*	This work
Plasmids		
pGB2Ts	*repA1*(Ts) derivative of pGB2 (8); *psi*-deleted resistance to streptomycin and spectinomycin	
pFC9	pGB2Ts carrying an *Eco*RI-*Pst*I 33-bp *dif* synthetic site inserted in the polylinker[a]	
pAMT6	pSC101 *repA1*(Ts) replication origin, psi^+, resistance to chloramphenicol	11
pFC13	pAMT6 carrying a 540-bp *Eco*RV fragment containing the $rpsL^+$ gene inserted at the *Sma*I site.	
pFC20	*psi* mutant derivative of pFC13 by deletion of a 890-bp *Nde*I-*Fsp*I fragment containing *psi* and part of *orfX*	
pFC24	pFC20 carrying a chromosomal 4.4-kb *Eco*RI-*Bam*HI *dif*-containing fragment from pBS12 (2) inserted in the polylinker	
pFC25	Δ*dif* derivative of pFC24 by replacement of a 58-bp *Dde*I-*Pfl*MI segment containing *dif* by a 8-bp *Dde*I-*Bam*HI-*Pfl*MI linker[b]	
pFC23	Same as pFC25 but with an insertion of a 2,775-bp *Bgl*II fragment of Tn*10* containing the Tet^r determinant inserted at the *Bam*HI site replacing *dif*	
pFC27	pFC13 with the 4.4-kb *Eco*RI-*Bam*HI *dif*-containing fragment from pFC24 inserted in the polylinker	
pFC27R	pFC27 having undergone an in vivo deletion between *dif* and *psi*, removing $rpsL^+$ plus the chromosomal region right of *dif* and creating the *pif* site	
pFC35	In vitro deletion of pFC23 between sites *Hin*dIII and *Bam*HI, removing 1.8 kb of chromosomal origin	
pFC36	pFC35 carrying a 217-bp PCR-amplified *pif*-containing fragment inserted at the *Hin*dIII site located in the Tn*10* fragment	
pFC66	pFC35 with a 3,710-bp *Hin*dIII fragment from pFC63 containing *psi* and the Sp^r Sm^r determinant inserted at the *Hin*dIII site	
pDJ2	pFC13 carrying a *Sma*I-*Fsp*I $lacI^q$-containing fragment (a gift from P. Polard) inserted at the *Nae*I site located between *cat* and *rpsL*	
pDJ1	pDJ2 with a 890-bp *Nde*I-*Fsp*I deletion removing *psi* and part of *orfX* (same as in pFC20)	
pIM6	pDJ2 with a 557-bp *Nae*I-*Fsp*I deletion removing *psi*	
pIM8	pDJ2 with a 1-bp insertion in the *Nde*I site creating an *orfX* frameshift mutation[c]	
pIM11	pDJ2 with a 333-bp *Nde*I-*Nae*I deletion eliminating part of *orfX*	
pAPT110	p15A replication origin vector carrying the $lacI^q$ gene and Kn^r and Sp^r Sm^r determinants	20
pIM9	pAPT110 with a 1,188-bp *Hae*III fragment from pFC13 containing the end of the *repA* gene, *orfX*, and the *psi* site from pSC101 inserted at the *Bsa*BI site	
pIM10	pIM9 with deletion of a 683-bp *Bam*HI-*Nde*I fragment of pSC101 origin, with removal of the end of the *repA* gene and the beginning of *orfX* and with conservation of *psi*	
pFC63	pIM10 with deletion of a 333-bp *Bam*HI-*Nae*I fragment, removing the rest of *orfX* still present in pIM10, with conservation of *psi*	
pFC65	pIM10 with deletion of a 1,717-bp *Nae*I fragment, removing 439 bp from pSC101 including *psi*, and 1,278 bp from pAPT110, with conservation of the *orfX* end	

[a] The two complementary oligonucleotides, 5′-GTTGGTGCGCATAATGTATATTATGTTAAATCTC-3′ and 5′-AATTCTGATTTAACATAATATACATTAT GCGCACCAACTGCA-3′, were mixed in Tris-EDTA buffer (22) at 6.25 μg/ml, heated at 94°C for 2 min, and then cooled slowly to 4°C in 5 h. The annealed oligonucleotides were then ligated to pGB2Ts digested with *Eco*RI and *Pst*I.

[b] pFC24 was partially digested with *Dde*I and then totally digested with *Pfl*MI. Fragments corresponding to full-size linear plasmids were separated on agarose gel (22), purified, and ligated with the hybridized oligonucleotides 5′-TCAGGATCCACTCG-3′ and 5′-GTGGATCC-3′.

[c] pDJ2 was digested by *Nde*I and self ligated, after the cohesive ends were filled by 3′ extension with Klenow DNA polymerase (22).

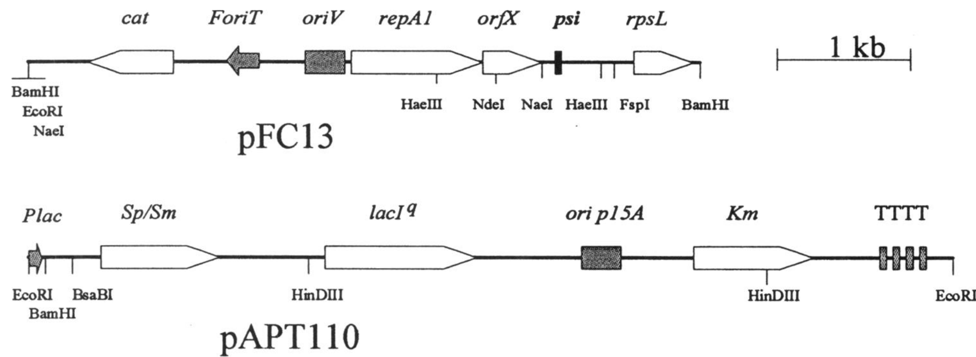

FIG. 2. Detailed maps of basic plasmids used in stability tests. pFC13 (this work) is a 5,278-bp plasmid harboring the following major elements (from left to right): the *cat* gene coding for a chloramphenicol acetyltransferase from transposon Tn*9*; *ForiT*, the origin of conjugative transfer from F factor; *oriV*, the replication origin of pSC101; *repA1*(Ts), a temperature-sensitive allele of the gene coding for pSC101 replication initiation factor; *orfX*, an open reading frame of unknown function probably coexpressed with *repA*; *psi*, the potential pSC101 target for XerCD recombinases; *rpsL*$^+$, the gene coding for the wild-type streptomycin-sensitive ribosomal protein S12. pAPT110 (20) is a 7,013-bp plasmid with the following characteristics (from left to right): *Plac*, the *lac* promoter; Sp/Sm gene, a gene conferring resistance to streptomycin and spectinomycin; the *lacI*^{q} gene coding for an overexpressed *lac* operon repressor; the p15A replication origin region; Km gene, a gene confering resistance to kanamycin; TTTT, a series of four transcription terminators from phage T4 gene 32.

at cell division and characterized by high instability in Rec$^+$ conditions (20).

The *psi* region strongly increased stability of pAPT110 by favoring multimer resolution. To analyze the role of *psi* in pAPT110 stability, various hybrid plasmids were constructed (Fig. 4). Plasmid pIM10 was obtained by inserting into pAPT110 the pSC101 *Nde*I-*Hae*III fragment from positions 6320 to 7094 on the pSC101 sequence (4), which contains *psi* and the distal part of *orfX*. The effect on stability was striking: the number of bacteria having lost pIM10 was less than 5×10^{-4} in either a Rec$^+$ host or a RecA$^-$ host. In the same conditions, pAPT110 was lost from 20 to 40% of Rec$^+$ bacteria (Fig. 4 and 5). We observed no multimers in pIM10 DNA prepared from Rec$^+$ bacteria, but multimers were present in the DNA of related less stable plasmids (Fig. 6). Thus, multimer resolution appears to be responsible for the improved stability of pIM10.

Plasmid pIM10 is as unstable as pAPT110 in either a XerC$^-$ or XerD$^-$ host (Table 2). This result strongly suggests that XerCD-mediated recombination at the *psi* site is directly involved in plasmid stabilization. In confirmation of this finding, the deletion of the small *Nae*I segment of pIM10, which removes the *psi* sequence, made the resulting plasmid, pFC65, as unstable as pAPT110 (Fig. 4 and 5). Clearly, however, accessory sequences are required for stabilization by *psi*: plasmid pFC63, analogous to pIM10 but with only the 441-bp *Nae*I-*Hae*III *psi*-containing fragment, is as unstable as pAPT110 in a Rec$^+$ strain (Fig. 4 and 5). That the *psi* core sequence is intact in pFC63 was verified by nucleotide sequencing. Thus, the active site for pIM10 multimer resolution is the *psi* core sequence plus 333 bp of accessory sequences starting 127 bp to its left side (and perhaps some material to its right). Note that both pIM10 and pFC63 plasmids made no multimer in a *recA1* host (Fig. 6). There is no indication that the *psi* site, in the pAPT110 context, could promote efficient intermolecular exchanges.

Analysis of the sequence surrounding *psi* revealed no obvious similarity with the *cer* accessory region (28), and the accessory factors, if any, are different from those required for *cer* since pIM10 is stable in an *argR* or *pepA* host (Table 2). Note also that our results clearly eliminated a requirement for OrfX by *psi*, since *orf* is disrupted in pIM10.

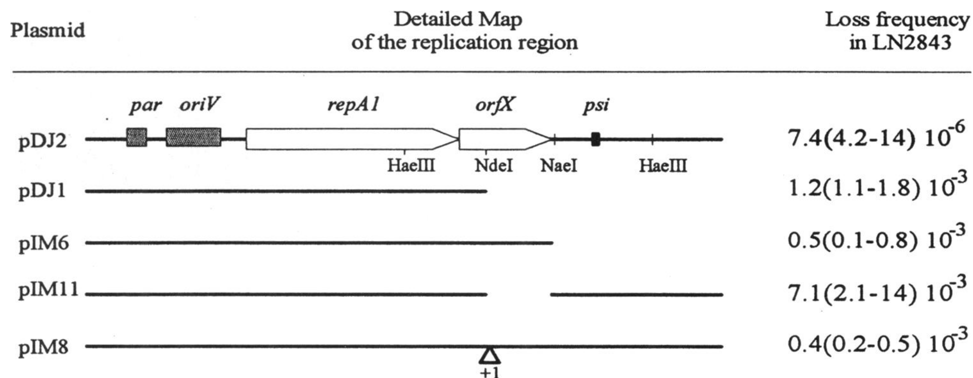

FIG. 3. Derivatives of pFC13 used in stability tests. The parental plasmid of this series, pDJ2, differs from pFC13 by insertion of the *lacI*^{q} gene (Table 1). Only the relevant pSC101 replication region is drawn: *par*, partition site; other symbols as described in the legend to Fig. 2. The positions of some useful restriction sites are also given. Plasmids pDJ1, pIM6, and pIM11 are deletion mutants of pDJ2, the conserved sequences being indicated by heavy lines; pIM8 is a +1 insertion mutation of *orfX*. The frequencies of Sm^r clones were determined in five clones for each strain. Average values and ranges (in parentheses) are given.

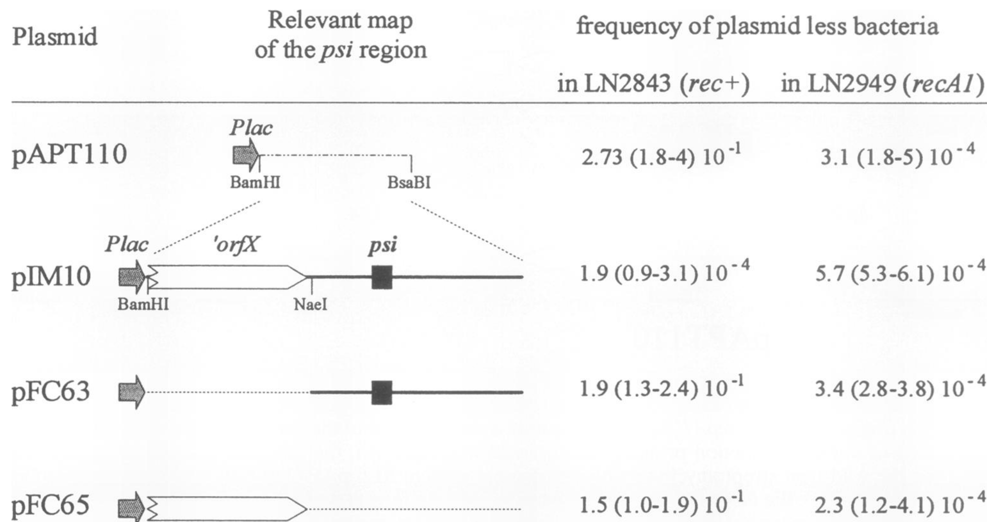

FIG. 4. Structure and stability of pAPT110 derivatives harboring various segments of the pSC101 *psi* region. In pIM10, the *psi*-containing fragment from pSC101 is a 774-bp *Nae*I-*Hae*III segment. This fragment has had the sequences indicated by the dotted lines in pFC63 and pFC65 deleted. For stability tests, colonies on L agar plus kanamycin (selective for plasmid presence) were inoculated into Luria medium and grown without selective pressure for about five generations. The cultures were then diluted, plated on L agar medium, and incubated overnight at 37°C. The frequencies of blue colonies (i.e., those resulting from multiplication of a plasmidless bacterium) were then determined in five clones for each strain. Average values and ranges (in parentheses) are given.

A hybrid *psi-dif* site functions in place of *dif* on the chromosome. The 4.4-kb *Bam*HI-*Eco*RI chromosomal fragment carrying *dif* was inserted into pFC13 to yield plasmid pFC27. In this plasmid, the *rpsL*$^+$ gene is flanked by *psi* and *dif*. When an *rpsL* strain carrying pFC27 was plated in the presence of streptomycin and chloramphenicol, the number of colonies that arose was close to 10^{-4}. These colonies harbored a plasmid with a deletion of the *psi-dif* interval and appear to have arisen from infrequent exchange between the *dif* and *psi* sequences, since no such colonies were obtained in a *xerC* strain. Sequencing these deletions revealed that they all (five examples) contained a hybrid *psi-dif* site which was flanked on one side by the pSC101 sequence and on the other by the chromosomal sequence. The hybrid sequence, named *pif*, has the same sequence as does *psi*, except for one nucleotide (Fig. 1). Interestingly, the rare exchanges forming *pif* have necessarily occurred between the XerD arms (6); this is the first example that the exchange may occur to the right of the central region (22a).

This recombinational exchange between *dif* and *psi* moti-

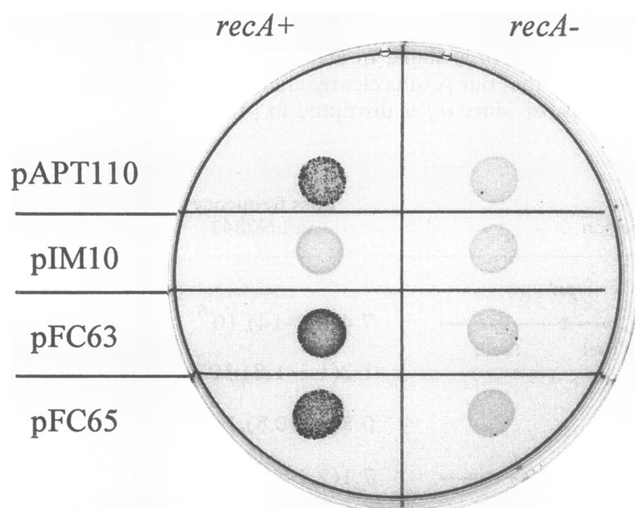

FIG. 5. Patch tests for stability of pAPT110 derivatives. The tested plasmids were carried by LN2843 (a *lacI* mutant) or by its *recA1* derivative LN2949. Cultures (10 μl) grown as described in the legend to Fig. 4 were diluted to about 10^5 bacteria per ml, spotted on X-Gal-containing plates, and incubated overnight at 37°C. The dark dots are colonies of β-galactosidase overproducers having lost the *lacI*^{q} carrier plasmids.

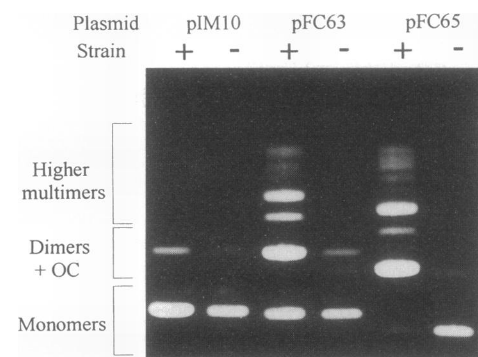

FIG. 6. *psi*-dependent plasmid monomerization. Cultures of Rec$^+$ and Rec$^-$ bacteria containing pIM10 (stably maintained in Rec$^+$ conditions) or its unstable deleted derivatives pFC63 (with the accessory region deleted) and pFC65 (with *psi* deleted) were grown overnight in Luria medium plus kanamycin, and plasmid DNA was extracted by alkaline lysis and electrophoresed as described in Materials and Methods. Lanes: +, carrier strain LN2843 (*rec*$^+$); −, carrier strain LN2949 (*recA1*). In the Rec$^+$ background, note the abundant proportion of dimers, trimers, and higher rank multimers for pFC63 and pFC65 but not for pIM10, which appears only as monomers. Note also the absence of multimers for all plasmids in Rec$^-$ conditions; under these conditions (0.4% agarose gels), dimers comigrate with relaxed monomers (OC).

TABLE 2. pIM10 stability in *xer* mutants[a]

Host strain	Avg no. of CFU (range)
LN2948 (*xerC*)	7.2×10^{-2} (2.1×10^{-2}–11.5×10^{-2})
LN2903 (*xerD*)	4.1×10^{-2} (3.4×10^{-2}–4.9×10^{-2})
LN2904 (*xerA = argR*)	1.3×10^{-4} (0.8×10^{-4}–1.6×10^{-4})
LN2905 (*xerB = pepA*)	0.9×10^{-4} (0.4×10^{-4}–1.3×10^{-4})

[a] The stability test was done as described in the legend to Fig. 4, except that the antibiotic used was spectinomycin instead of kanamycin. Three clones of each strain were tested; the values in parentheses are the extremes found.

vated us to examine the effect of substituting *pif* for *dif*. The genetic manipulations used to accomplish this are outlined in Fig. 7. We first deleted *dif* from the chromosome. Plasmid pFC23 was constructed from pFC20 (same as pFC27 but lacking the vector *psi* site to avoid exchanges between related sites) in two successive steps. First, we deleted a 58-bp *Pfl*MI-*Dde*I *dif*-containing segment and replaced it with a 8-bp linker harboring a *Bam*HI site. Then, at this *Bam*HI site we inserted the 2.8-kb *Bgl*II fragment from Tn*10* which contains the tetracycline resistance determinant. This Tc^r-tagged *dif* deletion was inserted into the chromosome by homologous recombination to give strain LN2772, whose structure was verified by PCR (data not shown). LN2772 displays the filamentation phenotype of Dif$^-$ mutants (Fig. 8) and does not allow high frequency integration into the chromosome of pFC9 (Table 3), a temperature-sensitive replication *psi*-deleted derivative of pSC101 (Table 1) which contains the *dif* site.

A similar procedure was followed to create a *dif*-deleted *pif*-harboring strain. Plasmid pFC36 was derived from pFC23 by cloning a 217-bp PCR product harboring the *pif* hybrid site into the *Hin*dIII site of the *tet* fragment. This Tc^r-tagged *pif* site was crossed into the chromosome to yield strain LN2799 (Fig. 7). The loss of the *dif* site in this strain was verified by PCR

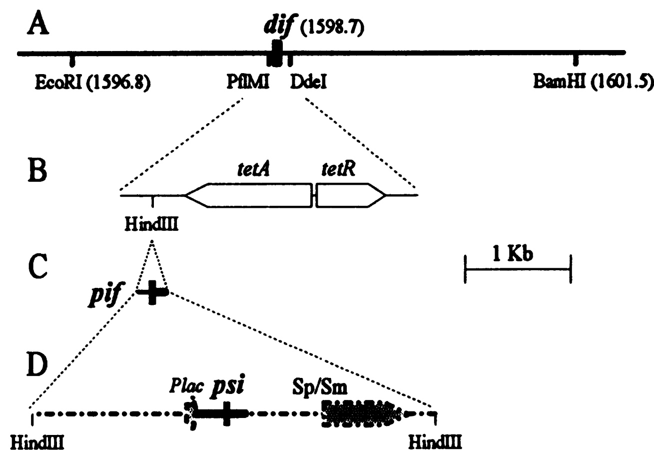

FIG. 7. Deletion of *dif* and its substitution by *pif* or *psi*. (A) Relevant physical map of chromosomal *dif* region (ordinates are from Rudd's physical map, 6th ed.; unpublished), the *Eco*RI-*Bam*HI fragment has been cloned into pFC13 to yield pFC27. (B) Structure of the Δ*dif*::*tet* substitution (LN2772). The short *Pfl*MI-*Dde*I fragment (58 bp) was replaced by a 2.9-kb *Bgl*II fragment from Tn*10* carrying the Tc^r determinant. (C) Structure of Δ*dif*::*pif* substitution (LN2799). A short *pif*-containing PCR fragment from pFC27R was installed at the *Hin*dIII site of the *tet* fragment. (D) Structure of the Δ*dif*::*psi* substitution (LN2974). A 3.7-kb *Hin*dIII *psi*-containing fragment from pFC63 was installed at the *Hin*dIII site of the *tet* fragment. The three sites display the same orientation in the chromosome (inverted with respect to Fig. 1).

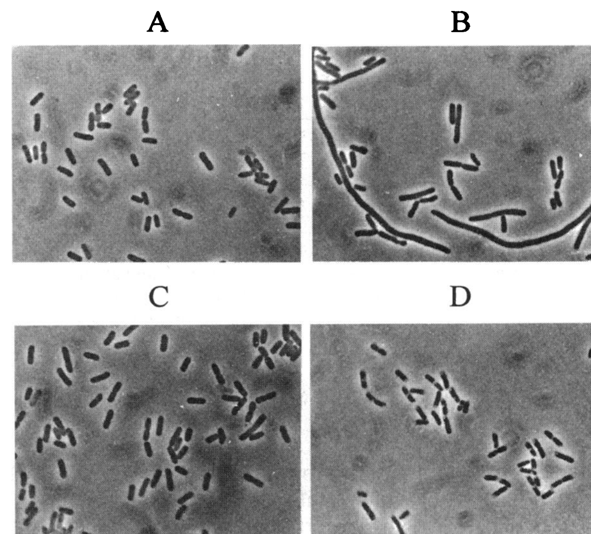

FIG. 8. Morphology of *dif*-substituted bacteria. Bacteria exponentially grown in Luria medium at 37°C were observed under phase contrast with a Leitz Ortholux II microscope and photographed. Magnification, ×2,300. (A) LN2666 (*dif*$^+$); (B) LN2772 (Δ*dif*); (C) LN2799 (Δ*dif*::*pif*); (D) LN2974 (Δ*dif*::*psi*).

analysis (data not presented) and by demonstrating that the *dif*-carrying plasmid pFC9 was integrated into the chromosome of LN2799 with a frequency nearly 1,000 times lower than that observed in the parental *dif*$^+$ strain (Table 3). LN2799 bacteria do not display the filamentation phenotype characteristic of *dif* mutants (Fig. 8) and do allow high frequency integration of a temperature-sensitive replication *psi*-containing plasmid, pFC13 (Table 3). Clearly, when inserted in the chromosome at the *dif* location, the *pif* site is active in site-specific intermolecular recombination and replaces *dif* functionally.

***psi* can also promote intermolecular recombination and replace *dif* on the chromosome.** The observation that the *psi*-containing vector pFC13 recombined into the *pif*-harboring LN2799 chromosome at a high frequency (Table 3) clearly demonstrated that *psi* in its normal context can promote intermolecular recombination and strongly suggested that *psi* and *pif*, whose sequences differ by only a single base pair, are functionally interchangeable. This led us to determine if the *psi* sequence, like the *pif* sequence, could also replace the *dif* site in the chromosome. As shown in Fig. 7, we constructed a Δ(*dif*)*psi*$^+$ strain by replacing the *dif* site with a sequence from pFC63 harboring *psi* plus the Sp/Sm gene (note that *psi* in pFC63 was inactive for exchanges between pFC63 molecules [Fig. 4 and 6]). The resulting strain, LN2974, was verified by PCR for the presence of *psi* and the absence of *dif* (data not shown). It was phenotypically Dif$^+$ (Fig. 8) and allowed maintenance of pFC13 (*psi*$^+$) at 42°C at a frequency 20 times higher than that of pFC9 (*dif*$^+$) or pGB2Ts (Table 3; the already higher degree of maintenance of pGB2Ts and pFC9 was due to the contribution of homologous recombination between plasmid-borne and chromosome-borne Sp/Sm genes). Thus, the *psi* site in the chromosome was recombinationally active and substituted for *dif*. We obtained similar results when the *psi* site plus the accessory sequence present in plasmid pIM10 was substituted in the chromosome for *dif* (data not shown). Thus, in this context, the activity of *psi* was not affected by the presence of the accessory region.

TABLE 3. Integration frequency of temperature-sensitive replication plasmids[a]

Host strain	Avg no. of CFU (range) on:		
	pGB2Ts (no site)	pFC9 (*dif*⁺)	pFC13 (*psi*⁺)
LN2666 (*dif*⁺)	1.9×10^{-5} (0.9×10^{-5}–3.8×10^{-5})	6.2×10^{-2} (2.0×10^{-2}–9.8×10^{-2})	9.6×10^{-5} (9.0×10^{-5}–10.8×10^{-5})
LN2772 (Δ*dif*)	3.6×10^{-7} (2.2×10^{-7}–5.6×10^{-7})	8.9×10^{-6} (8.0×10^{-6}–11.3×10^{-6})	1.3×10^{-5} (1.0×10^{-5}–1.6×10^{-5})
LN2799 (Δ*dif::pif*)	2.0×10^{-5} (0.6×10^{-5}–4.6×10^{-5})	1.1×10^{-4} (0.9×10^{-4}–1.1×10^{-4})	1.5×10^{-1} (0.7×10^{-1}–2.1×10^{-1})
LN2974 (Δ*dif::psi*)	1.4×10^{-3} (0.2×10^{-3}–2.3×10^{-3})	2.5×10^{-3} (1.4×10^{-3}–3.3×10^{-3})	4.6×10^{-2} (3.8×10^{-2}–5.9×10^{-2})

[a] Three clones of each strain, cultivated at 30°C in LB (19) with the relevant antibiotic (spectinomycin for pGB2Ts and pFC9 and chloramphenicol for pFC13), were plated at convenient dilutions either on L agar and incubated at 30°C or on L agar with the relevant antibiotic and incubated at 42°C. Average numbers of CFU at 42°C in the presence of antibiotic are given, with ranges indicated in parentheses. Note that certain plasmids tested share homology with certain host strains: pGB2Ts and pFC9 share the Sp/Sm interposon with LN2974, and pFC13 shares the *rpsL* region with all chromosomes.

DISCUSSION

We have shown that the *psi* site of pSC101 is proficient in site-specific recombination. However, the importance of this activity in pSC101 plasmid stability is limited, and multimers of a Δ(*psi*) pSC101 derivative are rare even in Rec⁺ bacteria. Our observations of the *psi* site and its derivative *pif* are consistent with their participation in XerCD-dependent recombination and emphasize the importance of the sequence context. A pair of *psi* sites is inactive (plasmid pFC63) or constrained and biased towards resolution (plasmid pIM10) when the site is harbored by plasmid pAPT110. It is unconstrained and able to promote intermolecular recombination when one of the partners is inserted in the chromosome at the position of *dif*, where it replaces *dif* in its normal physiological role (strain LN2974). Such versatile behavior has not previously been reported for this family of recombination sites.

As suggested by Sherratt and coworkers (23, 24), the role of accessory sequences and factors, characterizing constrained exchange reactions, is probably topological. These sequences facilitate the achievement of an active configuration between the sites, which is obtained only between sites belonging to the same molecule, hence the specificity towards resolution. Our observation that XerCD activity on a pair of *psi* core sequences can be shifted from constrained to unconstrained in response to the genetic context also points to a decisive role of local topology. The topological state of the DNA surrounding the sites may differ strongly between pAPT110 and the *dif* region, with recombination disfavored in the pAPT110 context but facilitated in the Tn*10 tet* fragment installed in place of *dif*.

The constraints on site-specific recombination need further exploration, and for the moment, the analysis of the pSC101 sequence activating *psi* in the pAPT110 context gives little clue to its mode of action. Preliminary data indicate that the very same pSC101 fragment which is active only in resolution when installed in pAPT110 favors multimerization when it is inserted into the *tet* gene of pBR322. Plasmid pBR322 is known for its special topological behavior compared with other plasmids of this family (7, 21). This reinforces the contention that local supercoiling controls *psi* activity. It is relevant to note here that such variable behavior has also been observed in the bacteriophage P1 Cre-*loxP* site-specific recombination system: the reaction is unconstrained in vitro but strictly resolutive in vivo (1).

An important result of the present work is the observation that the Dif⁻ phenotype is suppressed by integration into the chromosome of the related sites *psi* and *pif*. The substitution occurred at the exact position of the deleted *dif* site on the chromosome but with the site embedded in a sequence (the Tn*10 tet* region) unrelated to the normal ones of *psi* or *dif*. When these nearly identical sites integratively suppress the phenotype of the *dif* deletion, they may (strain LN2975) or may not (strain LN2974) carry the accessory region required for resolution in the pAPT110 system. This suggests that no accessory sequence is needed for *psi* or *pif* to fulfill the physiological operations requiring site-specific recombination in this region of the chromosome. This is also true for *dif* (5).

Recent work by Kuempel (13a) and Sherratt (22a) and coworkers indicates that translocation of *dif* to other positions on the chromosome (in the 32-min region and at *lac*, respectively) yields a Dif⁻ phenotype. Our present results may help to clarify the meaning of these observations. If *dif* were, like *psi*, sensitive to local sequence variations, an important factor for activity of the displaced *dif* sites should be the topological state of its insertion site, which was not known in these translocation experiments. A further factor is the probability of communication between the sites. If the physiological role of *dif* is to facilitate exchanges between sister chromosomes (whether fused in a dimer or not), the displaced *dif* sites must find each other at a high frequency after replication. This is perhaps achieved only in the terminus region where excisive homologous recombination reaches an extraordinarily high frequency, suggesting that exchanges between sister chromosomes might occur in this region in almost every generation (16).

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