Restriction of the activity of the recombination site *dif* to a small zone of the *Escherichia coli* chromosome

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The recombination site *dif* is the target on the *Escherichia coli* chromosome of the site-specific recombinases XerC and XerD. The *dif*/XerC-D system plays a role during the cell cycle, probably by favoring sister chromosome monomerization or separation. A phenomenon of regional control over *dif* activity, also analyzed in this issue, is demonstrated here by translocation of *dif* to a series of loci close to the normal locus. We found that the site is physiologically active only within a narrow zone around its natural position. Competence for *dif* activity does not depend on the sequence of the normal *dif* activity zone (DAZ), because $\Delta(dif)$ deletions larger than the DAZ result in Dif⁺ bacteria when *dif* is reinserted at the junction point. Although *dif* maps where replication normally terminates, termination of replication is not the elicitor. A strain with a large inversion that places *dif* and its surrounding region close to *oriC* remains Dif⁺, even when a Tus⁻ mutation allows replication to terminate far away from it. Preliminary data suggest the possibility that specialized sequences separate the competent zone from the rest of the chromosome. We suspect that these sequences are members of a set of sequences involved in a polarized process of postreplicative reconstruction of the nucleoid structure. We propose that this reconstruction forces catenation links between sister chromosomes to accumulate within the DAZ, where they eventually favor recombination at *dif*.

[Key Words: Site-specific recombination; *dif* site domain of activity; *E. coli* chromosome organization]

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The *Escherichia coli* chromosome harbors a recombination site, *dif*, that maps where replication normally terminates. Bacteria deficient for *dif*-specific recombination display decreased growth rate and colony forming ability and frequently form filaments with nucleoid partitioning defects (Blakely et al. 1991, 1993; Kuempel et al. 1991). Recombination between two *dif* sites is catalyzed by the XerC and XerD recombinases, which are also involved in the resolution of plasmid multimers (Blakely et al. 1991, 1993). It has been proposed that *dif* facilitates chromosome separation before partition, for instance, by resolving chromosome dimers that result from an odd number of exchanges between sister chromosomes (Blakely et al. 1991; Kuempel et al. 1991). This physiological role may involve frequent exchanges between *dif* sites belonging to sister chromosomes, fused or not. Because *dif* lies in the region of the chromosome replicated last (Louarn et al. 1994), a pair of *dif* sites (the normal target for a recombinase) is available only at termination. Consequently, site-specific recombination at *dif* should proceed most often between complete sister chromosomes. This implies that the spatial positioning inside the cell of two *dif* regions belonging to different

nucleoids should at least create no obstacle to, and perhaps could facilitate, exchanges between the sites.

The location of the *dif* site on the chromosome seems critical for its activity. A Dif⁻ phenotype results from translocations of *dif* to the *lac* operon at 8 min on the chromosome map, to a site near *oriC* (Leslie and Sherratt 1995), and even to a site within the terminus region, 118 kb to the left of its natural position (Tecklenburg et al. 1995). Nevertheless, these translocated sites were still able to recombine with a plasmid-borne *dif* site. Conversely, replacing *dif* at its natural position by either *psi*, the resolution site of plasmid pSC101, or *loxP*, the resolution site of bacteriophage P1 (in the presence of Cre resolvase), does not disrupt the Dif⁺ phenotype (Cornet et al. 1994; Leslie and Sherratt 1995). Therefore, the location of the recombination site seems more important for the chromosome than the nature of the site itself. Another remarkable feature of the region containing *dif* is a high frequency of excision by homologous recombination between direct repeats (Louarn et al. 1991). This phenomenon, called terminal recombination, is independent of termination of replication and of any unique sequence of the region, and is probably caused by inter-chromosomal exchanges (Louarn et al. 1994; J. Louarn, F. Cornet, J. Patte, and J.M. Louarn, unpubl.). Although *dif*-specific and excisive recombination processes are mech-

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anistically unrelated, they share the property of being elicited in the same chromosomal region. Whether the two recombination processes are under the same regional control and how this control is exerted, are the two major questions investigated in this paper.

Our data indicate that the competent zone for *dif* activity is restricted to a small region around its natural position, confirming the observations presented by Kuempel et al. (this issue). Deletion analyses indicate that no specific sequence-eliciting *dif* activity exists within the competent zone, thus showing that competence for *dif* activity is created by external factors. In addition, we demonstrate that replication termination does not determine the location of the competent zone. We have also begun to analyze the region of transition between the competent zone and the rest of the chromosome. The data suggest that the transition effect is mediated by specific sequences.

Results

The *dif* translocation system and criteria for *dif* activity

Most of the experiments are based on relocating *dif* by deleting a short fragment containing the *dif* site and then reinserting a *dif*-containing fragment at predetermined chromosomal positions. The net result is a translocation of the *dif* site. In all constructions, the final translocation consists of the insertion of a 1.6-kb chromosomal *dif* fragment into the *EcoRV* restriction site of a *tetA* gene belonging to either a Tn10 transposon or a Tn10 subfragment inserted previously at a desired position into the chromosome of $\Delta(dif)$ strain LN3061. An example of the genetic structures involved is shown in Figure 1. These constructions involved several steps of sequence replacements which were effected using an integration–excision vector described earlier (Cornet et al. 1994). The recombinant plasmids thus constructed are described in Table 1.

The physiological activity of a translocated *dif* site cannot be measured by a simple test reaction. Five characteristics differentiate a $\Delta(dif)$ strain from its wild-type counterpart: filamentation, slower growth rate, lower colony forming efficiency, induction of the SOS system, and low recombination between a *dif*-carrier plasmid and the chromosome (Kuempel et al. 1991; this work). All of these characteristics except SOS induction have been measured in this study. High frequency of *dif*-carrier plasmid integration was observed at all translocated *dif* sites (see Tables 2–4), indicating that all of these sites can undergo XerCD-catalyzed recombination. Although the presence of filaments is a sensitive criterium, their frequencies are somewhat variable and not easily quantified, as also noticed by Tecklenburg et al. (1995). The activity of a translocated *dif* site was in consequence evaluated by measuring growth rate and/or colony-forming ability in addition to microscopic observation. The site was taken to be physiologically inactive (*Dif*[−] phenotype) when generation times and colony-forming abil-

Figure 1. The *dif* translocation system. (A) Map of the *dif* region on the *E. coli* chromosome. Coordinates are given in kb and min (numbers in dotted circles) according to Rudd's map (K. Rudd, pers. comm.). The locations of the Tn10 and *tet* insertions used, as well as the size of the region where terminal recombination has been observed (Louarn et al. 1994; J. Louarn, F. Cornet, J. Patte, and J.M. Louarn, unpubl.), are indicated. (B) Two-step construction of the $\Delta(dif2600)::tet\Omega dif$ strain. The cloned *dif* chromosomal fragment has been modified by deletion of *dif* and insertion of *tet* in vitro (plasmid pFC75 of Table 1) and then recombined into the chromosome to yield strain LN3079. The 1.6-kb *dif*-containing fragment was eventually crossed into the chromosomal *tetA* gene using the delivery plasmid pFC72 as described in Materials and methods. (C) Construction and typical structure of a strain harboring a translocated *dif* site. The starting strain is LN3061, which harbors $\Delta(dif2600)\Omega Ap$ crossed into the chromosome using plasmid pFC73 (Table 1). The *tet* segments inserted at various positions along the chromosome (the *zdd355::tet* insertion shown here) were crossed into this strain by P1 transduction. The *dif* site was eventually inserted into the *tetA* locus using pFC72.

Table 1. *Plasmids used*

Plasmid	Description and References ^a
pFC9	plasmid pGB2T ⁺ carrying a synthetic 33-bp <i>dif</i> site (Cornet et al. 1994).
pFC20	an integration-excision vector used for substitution of chromosomal fragments (Cornet et al. 1994).
pLN135	pFC20 with a modified polylinker harboring <i>Nsi</i> I and <i>Hind</i> III sites ^b
pLN138	pLN135 carrying the 1561.4–1565.8 <i>Pst</i> I– <i>Kpn</i> I chromosomal fragment from pBS10 (Béjar and Bouché 1983) cloned in the polylinker
pFC68	pFC20 with the 2.8-kb <i>Bst</i> YI <i>tet</i> fragment from Tn10 (from pFC35; Cornet et al. 1994) inserted into the <i>Bam</i> HI site of the polylinker.
pLN139	pLN138 carrying the 2.8-kb <i>Bst</i> YI <i>tet</i> fragment from pFC68 cloned in the <i>Bam</i> HI site creating the <i>zdc310::tet</i> insertion at position 1563.0.
pFC72	pFC68 carrying the 1597.8–1599.8 <i>Eco</i> RV <i>dif</i> -containing chromosomal fragment (from pBS12; Béjar and Bouché 1983) cloned into the <i>Eco</i> RV site of the <i>tetA</i> gene. The two orientations of <i>dif</i> with respect to <i>tetA</i> exist (see Material and methods, Fig. 1).
pFC73	pFC24 (pFC20 carrying the 1596.8–1601.2 <i>Eco</i> RI– <i>Bam</i> HI <i>dif</i> -containing chromosomal segment; Cornet et al. 1994) deleted for the 1597.8–1600.4 <i>Eco</i> RV segment then replaced by a 2.6-kb Ω Ap <i>Sma</i> I fragment (from plasmid pKT254 <i>Ω</i> Ap; Fellay et al. 1987), to yield the Δ (<i>dif2600</i>) Ω Ap deletion-substitution (Fig. 1).
pFC75	pFC73 with a 2.6-kb <i>Hind</i> III deletion removing the Ω Ap interposon, replaced by the 2.8-kb <i>Bst</i> YI <i>tet</i> fragment from pFC68, to yield the Δ (<i>dif2600</i>):: <i>tet</i> deletion-substitution.
pFC85	pLN135 carrying the 1603.7–1611.7 <i>Kpn</i> I chromosomal fragment from pBS12 (Béjar and Bouché 1983) cloned in the polylinker.
pFC86	pFC85 with the 2.8-kb <i>Bst</i> YI <i>tet</i> fragment from pFC68 cloned in the <i>Bam</i> HI site at position 1607.6, creating the <i>zdd355::tet</i> insertion.
pFC89	pLN135 with the 1620.3–1623.6 <i>Kpn</i> I– <i>Bgl</i> II chromosomal fragment from Kohara phage 304 (Kohara et al. 1987) cloned into the polylinker ^c
pFC90	pFC89 with the 2.8 kb <i>Bst</i> YI <i>tet</i> fragment from pFC68 inserted in the <i>Bam</i> HI site at position 1622.4, creating the <i>zdd370::tet</i> insertions at position 1622.4 on Rudd's map.
pFC91	pFC89 deleted of the 1620.3–1622.4 <i>Kpn</i> I– <i>Bam</i> HI segment then replaced by the 1603.7–1607.6 <i>Kpn</i> I– <i>Bam</i> HI fragment from pFC85, to yield the Δ (<i>zdd355</i> – <i>zdd370</i>) deletion.
pFC92	pFC91 with the 2.2-kb <i>Bam</i> HI Ω Kn interposon (from plasmid pHP45 <i>Ω</i> Kn; Fellay et al. 1987) cloned into the fused 1607.6/1622.4 <i>Bam</i> HI site, to yield the Δ (<i>zdd355</i> – <i>zdd370</i>) Ω Kn deletion-insertion.
pFC93	pFC91 with the 2.8-kb <i>Bst</i> YI <i>tet</i> fragment from pFC68 cloned into the fused 1607.6/1622.4 <i>Bam</i> HI site, to yield the Δ (<i>zdd355</i> – <i>zdd370</i>):: <i>tet</i> deletion-insertion.
pFC94	pLN135 carrying a 20-kb chromosomal fragment (1603.7–1623.6) obtained by in vivo recombination between pFC92 and the <i>E. coli</i> chromosome (detailed in Materials and methods).
pFC95	pFC94 deleted for a 4.6-kb <i>BF</i> /II chromosomal segment then replaced by the 2.2-kb <i>Bam</i> HI Ω Kn interposon, to yield the Δ (1612.2–1616.8) Ω Kn deletion-insertion.
pFC97	pFC94 deleted for a 9.2-kb <i>Bam</i> HI– <i>Bgl</i> II chromosomal fragment then replaced by the 2.2-kb <i>Bam</i> HI Ω Kn interposon, to yield the Δ (1607.6–1616.8) Ω Kn deletion-insertion.
pFC101	pFC94 deleted for a chromosomal 10.1-kb <i>Bam</i> HI– <i>Bgl</i> II segment then replaced by the 2.8-kb <i>Bst</i> YI <i>tet</i> fragment, to yield the Δ (1612.2–1622.4):: <i>tet</i> deletion-insertion.
pFC102	pFC94 deleted for a chromosomal 9.2-kb <i>Bam</i> HI– <i>Bgl</i> II segment then replaced by the 2.8-kb <i>Bst</i> YI <i>tet</i> fragment, to yield the Δ (1607.6–1616.8):: <i>tet</i> deletion-insertion.
pFC103	pFC94 deleted for a chromosomal 4.6-kb <i>Bgl</i> II fragment then replaced by the 2.8-kb <i>Bst</i> YI <i>tet</i> fragment, to yield the Δ (1612.2–1616.8):: <i>tet</i> deletion-insertion.

^aFor sake of identification, chromosomal restriction site ordinates refer to the Ecomap6 of K. Rudd (pers. comm.).

^bThe two complementary oligonucleotides 5'-GATCATGCATGAAGCTT-3' and 5'-GATCAAGCTTCATGCAT-3' were mixed in TE buffer (Sambrook et al. 1989) at 6.25 μ g/ml, heated at 94°C for 2 min, and cooled slowly to 4°C in 5 hr. The annealed oligonucleotides were then ligated of pFC20 digested with *Bam*HI.

^cA 7.6-kb *Kpn*I fragment from Kohara phage λ 304 was first cloned in the *Kpn*I site of pLN135. The resultant plasmid pFC88 was digested with *Hind*III and *Bgl*II to remove a 4.3-kb fragment. Ends were blunted using T4 DNA polymerase (Sambrook et al. 1989), and the resulting fragment was self-ligated.

ities were similar to that of a reference Δ (*dif*) strain (34–36 min and ~65%, respectively, in Luria–Bertani medium (LB) at 37°C, compared with 25–26 min and >99% for the wild-type strain LN2666 in the same conditions).

The *dif* translocation system has been validated by showing that insertion of the *dif*-carrying 1.6-kb fragment into the *tet* determinant of a Δ (*dif2600*)::*tet* deletion (LN3080 in Fig. 1) largely restores the Dif⁺ pheno-

type, that is, normal generation time (Table 2, below), considerable improvement of the efficiency of microcolony formation (Fig. 4, below), and strong reduction of filamentation despite a cell-size dispersion larger than in the Dif⁺ parent (data not shown). Because all strains with a translocated *dif* site have undergone the Δ (*dif2600*) deletion and harbor the same *dif* fragment inserted into the *tet* segment, as in LN3080, the behavior

Table 2. Expression of the *Dif* phenotype in various *dif* translocations

Strain	Relevant genotype	Filaments	Doubling time	Efficiency of plating (%)	Integration of pFC9	<i>Dif</i> phenotype
LN2666	(+)	A	25	>99	$6.4 \cdot 10^{-2}$	+
LN3079	$\Delta(dif2600)::tet$	F	36	60	$2.3 \cdot 10^{-5}$	–
LN3080	$\Delta(dif2600)::tet\Omega dif$	A	25	96	$9.6 \cdot 10^{-2}$	+
LN3061	$\Delta(dif2600)\Omega Ap$	F	35	65	$1.1 \cdot 10^{-4}$	–
LN3320	LN3061 <i>zdc310::tet</i> Ωdif	F	35	N.D.	$5.0 \cdot 10^{-2}$	–
LN3161	LN3061 <i>zdd355::tet</i> Ωdif	R	26	88	$1.7 \cdot 10^{-1}$	+
LN3162	LN3061 <i>zdd370::tet</i> Ωdif	F	36	60	$8.1 \cdot 10^{-2}$	–
LN3046	LN3061 <i>zde381::Tn10</i> Ωdif	F	35	N.D.	$6.1 \cdot 10^{-2}$	–
LN3093	LN3061 <i>zde395::Tn10</i> Ωdif	F	37	N.D.	$5.9 \cdot 10^{-2}$	–
LN3092	LN3061 <i>zde406::Tn10</i> Ωdif	F	37	N.D.	$6.2 \cdot 10^{-2}$	–

The general procedures for strain construction are described in Materials and methods and Fig. 1. Plasmids used for *dif* translocation are listed in Table 1. Filamentation was observed by phase-contrast microscopy: (F) Frequent filaments, as in $\Delta(dif)$ strain LN3061; (R) rare filaments; (A) absence of filaments. Doubling times (in min, averages of two determinations) were measured in standardized conditions of exponential growth in LB medium at 37°C. Percentages of bacteria spread on agar slides giving rise to microcolonies were determined as described in Materials and methods; values indicated are the averages of several determinations, corresponding to the fate of several thousands bacterial cells. Integration frequencies of pFC9 are the means of four determinations of the ratio of colony-forming units at 42°C to those at 30°C on streptomycin plates. (N.D.) Not done.

of this strain is taken as the reference. Importantly, we found throughout this study that regardless of the location of *dif*, the orientation of the *dif*-containing fragment in the chromosome did not affect the *Dif* phenotype (data not shown).

Size of the zone competent for *dif* activity

We have translocated *dif* into six sites within the terminus region (Fig. 1). One translocation site mapped 37 kb to the left of *dif*, into *zdc310::tet* (LN3320), and the five others to its right, *zdd355::tet* (LN3161), *zdd370::tet* (LN3162), *zde381::Tn10* (LN3046), *zde395::Tn10* (LN3093), *zde406::Tn10* (LN3092), at 8, 23, 34, 48, and 59 kb, respectively, from *dif*. These insertions have no effect on growth rates or on efficiency of colony formation when introduced in LN2666 (generation times close to 25 min and colony formation efficiency >99%, whatever the insertion; data not shown). Translocation of *dif* at all of these positions, except *zdd355*, resulted in a clear *Dif*[–] phenotype (Table 2, below). Only the insertion of *dif* at *zdd355* restored a *Dif*⁺ phenotype, with generation time and plating efficiency at near wild-type levels, and partial but significant decrease of filament

frequency. Thus, *dif* can fulfill its physiological role when translocated 8 kb to the right of its natural position but not when it is translocated farther away to its right, or at 37 kb to the left. The normal competent zone for *dif* activity is therefore relatively narrow on the chromosome, consistent with the results presented by Kuempel et al. (this issue).

Dif activity does not require termination of replication in its vicinity

During normal replication, termination takes place at, or to the immediate left of, the pause site *psrA* (= *terC*) which is very close to *dif* (Fig. 1; Louarn et al. 1994). The clockwise moving replication fork probably stalls at *psrA* for a short while at the end of most replication cycles. To investigate the role of replication fork pausing as a possible elicitor of *dif* activity, *psrA* has been removed by a 15-kb deletion to yield strain LN3167. This strain is *Dif*⁺ (Table 3), as are strains having undergone deletions of the *tus* locus (data not shown), which controls site-specific replication pausing (Hill et al. 1988; Khatri et al. 1989; Lee et al. 1989). In strain LN3034 (Louarn et al. 1994), the region where replication termi-

Table 3. The *Dif* phenotype in strains modified for the replication fork trap

Strain	Relevant genotype	Filaments	Doubling time (min)	Integration of pFC9	<i>Dif</i> phenotype
LN2666	(+)	A	25	$6.4 \cdot 10^{-2}$	+
LN3167	$\Delta(zdd355-zdd370)\Omega Kn$	A	26	$7.8 \cdot 10^{-2}$	+
LN3034	$\Delta(1398-1402)\Omega psrA^*-Ap$	A	27	$7.1 \cdot 10^{-2}$	+
LN3085	LN3034 $\Delta(dif2600)::tet$	F	35	$2.7 \cdot 10^{-5}$	–

Same legend as for Table 2. The construction of strain LN3034 has been described elsewhere (Louarn et al. 1994); ordinates refer to the endpoints of the chromosomal segment substituted with the additional replication terminator. LN3085 is a derivative of LN3034 constructed by transducing the Tet^r determinant of strain LN3079 (Table 2).

Table 4. Effect of deletions of the competent zone on dif activity

Strain	Relevant genotype	Filaments	Doubling time (min)	Efficacy of plating (%)	Integration of pFC9	Dif phenotype
LN2666	(+)	A	25	>99	$6.4 \cdot 10^{-2}$	+
LN3073	$\Delta(zdc235-zdf327)::tet$	F	45	70	$1.5 \cdot 10^{-4}$	-
LN3076	$\Delta(zdc235-zdf237)::tet\Omega dif$	A	40	94	$1.5 \cdot 10^{-1}$	+
LN3359	$\Delta(zdc310-zdd370)::tet$	F	34	68	$2.2 \cdot 10^{-5}$	-
LN3363	$\Delta(zdc310-zdd370)::tet\Omega dif$	A	28	95	$5.7 \cdot 10^{-2}$	+
LN3074	$\Delta(dif-zde381)::tet\Omega dif$	A	28	95	$1.2 \cdot 10^{-1}$	+
LN3215	$\Delta(dif2600)\Omega Ap$	R	27	94	$7.8 \cdot 10^{-2}$	+
	$\Delta(zdd355-zdd370)::tet\Omega dif$					
LN3169	$\Delta(dif2600)\Omega Ap$	F	38	66	$1.1 \cdot 10^{-1}$	-
	$\Delta(zdd355-zdd370)\Omega Kn$					
	$zde381::Tn10\Omega dif$					

Same legend as for Table 2. Strains LN3073 and LN3076 are derived from PK2035 (see Materials and methods). These strains grow poorly even when rendered Dif⁺. This is probably a consequence of the large deletion eliminating useful loci in addition to *dif*.

nates has been displaced to a position ~200 kb from *dif* by inserting an additional pause site. LN3034 is phenotypically Dif⁺ (Table 3), and its $\Delta(dif2600)::tet$ counterpart displays a Dif⁻ phenotype (strain LN3085, Table 3). Thus, the ability of the *dif* site to fulfill its role in the cell cycle does not require that termination of replication occur in the immediate vicinity of the site.

Dif is active even if replicated early

Strain LN850 (Louarn et al. 1985) carries a large chromosomal inversion attributable to recombination between two inverted copies of IS5, IS5F at 29 min (1404 kb on Rudd's map) and IS5T at 78 min (3674 kb). In this strain, the *dif* site lies 11 min from *oriC* (Fig. 2). Although the inversion is relatively harmless per se, strain LN850 displays a rich medium sensitivity (Rms) caused by the

presence of the temperature-sensitive *dnaA46* mutation (why the addition of these two genetic alterations results in a Rms phenotype is not known). The Rms phenotype is nevertheless a convenient indicator of the presence of the rather unstable rearrangement (spontaneous Rmr derivatives are found at frequencies above 10^{-3}). Previous analyses have shown that the replication arms are very unequal in this strain, because terminators that normally inhibit forks moving clockwise (*psrA*=*terC*, *psrB*=*terA*, *psrC*=*terF*) now inhibit forks moving counterclockwise after a short transit from *oriC* and early replication of *dif* (Fig. 2), so that the meeting of forks moving in opposite directions occurs around position 50 min on the map in the Tus⁺ conditions, in the vicinity of *psrC* (de Massy et al. 1987). In this strain *dif* is replicated in the first 15% of a replication cycle, which is ~1.5 times longer than normal. Because the counterclockwise moving forks pause for a while at *psrA*, in the immediate vicinity of *dif* (de Massy et al. 1987), the possibility that fork arrest elicits *dif* activity can still be considered.

Exponentially growing LN850 bacteria display no filamentation (Fig. 3A) or any sign of SOS induction (actually, the amount of RecA protein per cell is about one-third the normal level; P. Gamas and J.-M. Louarn, unpubl.). Strain LN3213 is derived from LN850 by introduction, through phage P1-mediated transduction of the $\Delta(dif2600)::tet$ deletion. This strain grows very poorly, remains Rms (but Rmr revertants, probably attributable to the restoration of the normal gene order, take over even more rapidly than in LN850), and is strongly prone to filamentation (Fig. 3B).

A $\Delta(tus)::Ap^r$ mutation (Louarn et al. 1994) was introduced into LN850 by transduction to yield strain LN3214, in which the absence of *tus* sequences was verified by PCR (data not shown). Chromosome replication was analyzed by marker frequency measurements in LN3214. The data (not shown) indicated no evidence for fork arrest at any of the *psr* sites. Although the inverted part of the chromosome appeared to be replicated at a reduced velocity in the direction opposite to normal, the

Figure 2. The large Inv(29–78) inversion. This simplified map of the *E. coli* chromosome gives the positions of the endpoints of the rearrangement (the two IS5s) and illustrates its major consequences for chromosome replication: new location of *dif* close to *oriC*, and reorientation of the replication terminator sites. Convergent arrows indicate the probable zones of replication fork meeting in Tus⁺ (~50 min) and Tus⁻ conditions (not known precisely).

Figure 3. Effect of the deletion of *dif* on bacteria carrying the large inversion. Bacteria growing exponentially in Vogel and Bonner E minimal medium at 30°C were observed under phase-contrast microscopy with a Leitz Ortholux microscope and photographed. Original magnification, 2300 $\times$. (A) LN850 (Inv29–78); (B) LN3213 (Inv29–78 Δdif); (C) LN3214 (Inv29–78 Δtus); (D) LN3224 (Inv29–78 $\Delta dif \Delta tus$).

meeting of forks moving in opposite directions occurred at a great distance from *dif*, ~ 1 Mb away. No particular tendency to filamentation was detected (Fig. 3C) nor was there any correction of the Rms phenotype. The triple mutant Inv(29–78) $\Delta(dif2600)::tet \Delta(tus)::Ap^r$ (LN3224) behaved similarly to LN3213, showing extensive filamentation (Fig. 3D) and reduced colony-forming ability.

Obviously, *dif* fulfills its physiological role in the inverted strains, even when termination occurs far away from it. Not only do these data definitely rule out the possibility that termination of replication is an enhancer of *dif* activity, but they also indicate that the timing of *dif* replication is unimportant for its physiological activity.

dif activity is independent of any sequence in the normal competent zone

The regional control over *dif* activity could be attributable to specific elicitor sequences located within the competence zone. If this were true, the reinsertion of a *dif* locus into chromosomes deleted for the *dif* competence region should never give rise to Dif⁺ strains. Two large deletions of the *dif* region have been assayed (Table 4), a 60-kb deletion between positions *zdc310* and

zdd370 with a *tet* fragment joining the deletion endpoints (strain LN3359), and a 155-kb deletion between positions *zdc235* and *zdf237* eliminating 57 kb to the left of *dif* and 98 kb to its right with a Tn10 joining the deletion endpoints (strain LN3073; Kuempel et al. 1991). Both strains are $\Delta(dif)$ and form filaments at high frequency. When the *dif*-containing fragment was inserted into the *tet* or Tn10 sequences (strains LN3363 and LN3076), filaments disappeared and doubling times became lower than those of the parent strains (Table 4). Moreover, the efficiency of colony formation was restored to normal (Table 4). These bacteria clearly display a Dif⁺ phenotype. These experiments lead to two important conclusions: (1) Inactive *dif* sequences at positions on either side of the competent zone can become active provided the intervening DNA (i.e., the competent region itself) is deleted; and (2) the competent region contains no unique sequence required for the activity of the translocated sites.

A translocated inactive *dif* site can be activated by nearby deletions

Consistent with the above observations, we observed repeatedly that deletion of the region separating an inactive translocated *dif* site from its natural position results in a Dif⁺ phenotype. This was found, for instance, for *dif* translocations at positions *zde381* (strain LN3074 in Table 4) and *zde395* (data not shown), suggesting that such deletions removed determinants rendering the translocated site inactive. To analyze this phenomenon of activation by deletion, we concentrated on the region between *zdd355* and *zdd370* *tet* insertions. The presence of *dif* at position *zdd370* (strain LN3162 in Table 2) does not suppress the effects of the $\Delta(dif2600)::Ap^r$ deletion, whereas its presence at position *zdd355* (strain LN3161 in Table 2) is suppressive. We deleted the material between the two *tet* insertions and inserted *dif* within the *tet* sequence tagging this 14.7-kb deletion. The resulting strain (LN3215 in Table 4) appeared Dif⁺ to the same extent as LN3161 (*dif* at *zdd355*, Table 2). Thus, the nonfunctionality of *dif* inserted at *zdd370* is determined by sequences of the *zdd355*–*zdd370* interval. Activation of *dif* by this deletion is only a local effect. When the *zdd355*–*zdd370* region was deleted from chromosomes harboring *dif* at the *zde381::Tn10* position, the resulting strain (LN3169 in Table 4) remained Dif⁺, although the very same translocated *dif* site was activated by the larger (*dif*–*zde381*) deletion (strain LN3074 in Table 4).

Deletion analysis of the transition from inside to outside the competent zone

One possible explanation for the above results is that specialized border sequences act as barriers separating the zone competent for *dif* activity from the rest of the chromosome. In this view, strain LN3215 (Table 3; Fig. 4A), which is $\Delta(zdd355\text{--}zdd370)::tet\Omega dif$, is Dif⁺ because the deletion of the *zdd355*–*zdd370* interval had

Figure 4. The search for a fixed limit to the DAZ. Presented are the effects of various deletions in the *zdd355*–*zdd370* region (1607.6–1622.3 kb on Ecomap6; K. Rudd, pers. comm.) on the physiological activity of translocated *dif* sites. (A) Physical map of the *zdd355*–*zdd370* region, showing the positions of the *Bam*HI and *Bgl*III restriction sites used for creating the rearrangements, and the natural position of *dif*. (B) The various deletions and their growth characteristics. Segments remaining to the left of the translocated *dif* site are numbered with reference to A. These constructions were created and inserted into the chromosome, as described in Materials and methods. Plasmids (Table 1) involved as intermediates in the constructions: pFC97 for LN3219; pFC95 for LN3220; pFC103 for LN3286; pFC102 for LN3288; and pFC101 for LN3290.

eliminated the right (in reference to Fig. 1) border of the *dif* activity domain, thus allowing an extension of this domain to *zdd370* and beyond. To determine more precisely the map position of the border, partial deletions of the *zdd355*–*zdd370* interval have been constructed, with replacement of the excised fragment by the *tet* segment or a *Kn*^r segment. The deleted segments are numbered I–III in Figure 4A. All strains presented in Figure 4B carried a $\Delta(dif2600)::Ap^r$ deletion plus a *dif* site translocated either into the *tet* fragment tagging at the deletion or at the *zdd370::Tn10* position. The Dif⁺ phenotype of strains LN3286 and LN3290, in which the *dif* site is located to the immediate right of segment I, indicates that the region of *dif* activity extends at least 12 kb to the right of the natural position of *dif*. Therefore, the transition from inside to outside the competent zone must occur within segment II or III. Deletion of segment II (or of I + II) when *dif* is inserted at position *zdd370* (strains LN3220 and LN3219) allows some improvement of growth parameters compared with the situation in the

undelimited strain strain LN3162 (see also Table 2) but not to the level observed when the whole region is deleted (strain LN3215, see also Table 4). This suggests that segment III also contributes to the lack of functionality of *dif* at *zdd370* when the whole region is present. One possible explanation could be that the hypothetical right barrier limiting the *dif* activity zone (DAZ) is fully set up only when segments II and III are present. The observation that *dif* can display a partial activity (Dif^{+/-} phenotype) was a surprise. Nevertheless, these preliminary data are compatible with the proposed existence of specialized border sequences, even though they raise the possibility that the barrier effect involves rather complicated structures.

Discussion

Our data, combined with information published previously (Leslie and Sherratt 1995; Tecklenburg et al. 1995) and that presented in Kuempel et al. (this issue), confirm

that the physiological functions of *dif* can be achieved only when the site is situated within a narrow zone (perhaps 20–30 kb) around its natural position, even though it is able to recombine with a plasmid-borne *dif* site whatever its chromosomal location. This narrow competent zone is termed DAZ in the following. The main features of this position effect can be summarized as follows (1) The replication process is not directly involved. Although *dif* is normally present in the region where replication terminates, its activity is unrelated to termination of chromosome replication and does not require that the site is replicated at a precise time during the cell cycle. (2) The genetic content of the region competent for *dif* physiological activity is unimportant. Once the normal region competent for *dif* activity is deleted, a new competent region takes the place of the old one, implying that the competence is determined by factors external to the zone. (3) Deletion of the transition region between the DAZ and the noncompetent adjacent region does not inhibit the regional control over *dif* activity but, rather, seems to allow the extension of this control to positions normally incompetent. The transition region is therefore not an elicitor of competence but, rather, a barrier to propagation of competence. The fact that competence remains restricted to a rather small domain when a deletion has modified the original DAZ is suggestive of a plurality of sequences having the same potential as the transition region. (4) The transition from inside the domain of competence to outside on the right of the DAZ seems to involve specialized sequences. Dissection and reconstruction experiments are aimed at identifying these specialized sequences and should provide a more complete view of the transition region.

Strikingly, the observations and rules established on the DAZ also hold for terminal recombination (Louarn et al. 1994). The normal TRZ (terminal recombination zone) occupies a small fraction of the chromosome that overlaps the DAZ and is susceptible to the same kinds of chromosome rearrangements. For instance, strains deleted for the normal TRZ still display excise hyper-recombination at the junction of the deleted segment, and termination of chromosome replication does not appear to be a controlling factor (Louarn et al. 1994). Terminal recombination is dependent on RecBC(D), a fact implying that, in the experimental system we use, terminal recombination is an interchromosomal event (J. Louarn, F. Cornet, J. Patte, and J.M. Louarn, unpubl.). It also displays a polarization on both sides of the DAZ, which is consistent with a frequency of initiation events increasing gradually on both sides of the DAZ and culminating within this region (J. Louarn, F. Cornet, J. Patte, and J.M. Louarn, unpubl.).

How can we explain the restriction of *dif* activity to a narrow zone of competence, and what is the phenomenon that, occurring preferentially within the DAZ but not depending on unique sequences in this zone, facilitates terminal recombination and makes *dif*-specific recombination physiologically active? Localized intercatenation of sister chromosomes, which could in turn favor local interchromosomal recombination, is an obvi-

ous candidate. Surprisingly, termination of replication, which has been proven to favor intercatenation in some systems such as SV40 replication (Sundin and Varshavsky 1981), is not an elicitor of either *dif* activity (this work) or terminal recombination (Louarn et al. 1994), although both phenomena occur in the normal termination region. The model proposed for terminal recombination involved nucleoid organization (Louarn et al. 1994). We postulated that nucleoid reconstruction after replication occurs in the same direction as normal replication, in a sequential process involving specialized and ordered sequences. The model implied the existence of two polarized organization units along each replication arm, with a region (named the nucleoid terminus) separating these two units near the terminus. It might be that catenation links are progressively chased and accumulate in the nucleoid terminus as nucleoid reconstruction proceeds along the two still intercatenated sister chromosomes. Associated with decatenation or otherwise, interchromosomal exchanges by homologous recombination would be favored in the region, which can be considered as a zone of synapsis between sister chromosomes. This model can also provide the framework for an explanation of the restricted zone competent for *dif* activity. In this view, the DAZ is the nucleoid terminus, and the sequences involved in the transition between the DAZ and the rest of the chromosome should be the last organizing sequences of each organization unit. Also, a multiplicity of sequences involved in structuring the nucleoid makes it simple to envisage the creation of a new competent zone for *dif* activity after deletion of the original one. There must always be a zone where catenation links accumulate between the two rearranged organization units.

Interestingly, the existence of a fixed and polarized nucleoid organization has already been proposed to explain the seemingly unrelated observation that regions flanking the terminus (and spanning 20%–30% of the chromosome) are refractory to genetic inversions [non divisible zones (NDZ)s; Rebollo et al. 1988]. Recent explorations (M. Guijo, V. François, J. Patte, J.M. Louarn, and J.E. Rebollo, in prep.) have revealed that the NDZs are not continuous but rather that they encompass a series of domains, each refractory to inversion. We suspect that these domains are involved in the process of polarized nucleoid organization that we postulate here, and it may appear in the future that the DAZ, the TRZ, and the zones refractory to inversion (NDZ) are created by the same features of nucleoid organization. In the case of the large 29–78 inversion used in this work, one endpoint, the IS5F at 29 min, is probably located in a region linking two inversion-refractory domains, so that the crucial features of nucleoid organization might have been preserved after inversion, even though this 10- to 20-kb linking region has been replaced by a 2000-kb one. This should not modify the postulated process of catenation link migration, at least along the chromosomal arm that has conserved its normal organization. Hence, the region competent for *dif* activity would not be expected to be modified by the enormous chromosome rearrangement.

Materials and methods

Strains and plasmids

Plasmids used and their constructions are presented in Table 1. All strains derived from LN2666, a spontaneous streptomycin-resistant mutant of CB0129 (F^- W1485 *leu* *thyA* *deoB* or *C* *supE*; Bird et al. 1972), except LN3073, which is a spontaneous streptomycin-resistant mutant of PK2035 (Kuempel et al. 1991), and LN850, which is derived differently from CB0129 (Louarn et al. 1985). LN3079 and LN3061 are LN2666 derivatives made $\Delta(dif2600)::tet$ and $\Delta(dif2600)::Ap^r$ by homologous recombination with plasmid pFC75.1 and pFC73, respectively (integration then excision). The *zde381::Tn10*, *zde395::Tn10*, and *zde406::Tn10* insertions from strains LN2035, LN1954, and LN1953 (Louarn et al. 1994) were transferred by phage P1-mediated transduction into LN3061. The $\Delta(zdc310-zdd370)::tet$ and $\Delta(dif-zde381)::tet$ deletions were created by homologous recombination using the “*tes* and *tek*” system (François et al. 1987).

Media and general procedures

All *in vivo* experiments were carried out in LB medium (Miller 1992), except for LN850 and its derivatives, which were grown in M9 glucose medium or Vogel and Bonner E minimal medium as described previously (Louarn et al. 1985). Antibiotic concentrations used were 200 μ g/ml of streptomycin, 20 μ g/ml of chloramphenicol, 10 μ g/ml of tetracyclin, 50 μ g/ml of ampicillin, 50 μ g/ml of spectinomycin, and 25 μ g/ml of kanamycin. All plasmid constructions involved standard procedures (Sambrook et al. 1989; Table 1).

Doubling time and plating efficiency measurements

Generation times during exponential growth were determined on bacteria incubated in LB medium at 37°C with vigorous agitation for at least 4–6 hr and diluted into prewarmed LB medium to keep OD₅₄₀ nm below 0.2 at initial measurement. Plating efficiency was monitored by phase-contrast examination, at magnification 400 $\times$ with a Nikon Labophot-2A microscope, of bacteria growing on microscope slides spread with 0.6 ml of soft LB medium (Miller 1992), after incubation for 3 hr at 37°C in a wet chamber. The fate of at least 500 individual cells was followed for each determination. Plating efficiency is expressed as the percent of spread bacteria giving rise to microcolonies. Measuring the dynamic equilibrium between viable and inviable bacteria in an expanding population, this test gives reproducible results if growth conditions, in particular aeration, are kept as standardized as possible before spreading the bacteria on the slide.

Test for the presence of *dif* on the chromosome

The presence and functionality of a translocated *dif* site was tested by determining the efficiency of rescuing of plasmid pFC9 at 42°C, a *dif*-carrier replication-thermosensitive pSC101 derivative, as described previously (Cornet et al. 1994). Integration frequencies given in Tables 2, 3, and 4 are the average of four independent measurements.

Precise substitution of chromosomal segments

Use of plasmids of the pFC13 family for substitution of chromosomal segments had been described previously (Cornet et al. 1994). Chromosomal fragments are first cloned in pFC13-derived vectors and then modified to get the desired construction

(see Table 1). Transfer into the chromosome of the various constructions was performed by a two-step procedure. First, plasmid integration into the chromosome is selected by plating on chloramphenicol-containing medium at 42°C; second, plasmid excision and loss are selected by plating on streptomycin-containing medium at 42°C. The cured colonies are then screened for the desired antibiotic resistances. For example, *dif* translocations involved use of plasmids pFC72.1 and pFC72.2, which carry the Ωdif fragment inserted into the *Tn10 tetA* gene in either orientation (Fig. 1), and the success of transfer of *dif* into preexisting chromosomal *Tn10* or *tet* insertions was monitored by screening *Sm*^r derivatives for loss of tetracyclin resistance.

In vivo cloning of the *zdd355–zdd370* region

We took advantage of plasmid pFC92, which was constructed *in vitro* and carries the regions left of *zdd355* and right of *zdd370* joined by a *Kn*^r interposon (Table 1). LN2666 bacteria carrying this plasmid integrated into the chromosome (*Cm*^r at 42°C) were grown at 42°C in LB medium without antibiotics, and the rare plasmid molecules liberated by excision from the chromosome were prepared by standard alkaline lysis technique. Among the excision events, those taking place in the region not used for integration should result in plasmids harboring the wild-type *zdd355–zdd370* region. The plasmid preparation was used to transform strain LN2521 [CB0129 $\Delta(tus)\Omega Ap$; Louarn et al. 1994], with selection for resistance to chloramphenicol and then screening for kanamycin sensitivity. Among 67 *Cm*^r transformants, 4 were *Kn*^r and all of these harbored the desired plasmid, pFC94, which carries the 1602- to 1624-kb chromosomal segment.

Construction of deletions in the *zdd355–zdd370* region

Various segments, numbered I–III in Figure 4A, have been deleted from the pFC94 plasmid by partial digestions with *Bam*HI or *Bgl*II and replaced by either the *tet* segment or a *Kn*^r determinant. The tagged deletions have been eventually inserted into the chromosome, as described above. To ascertain the presence in the chromosome of modified *zdd355–zdd370* regions, these regions were picked up from the chromosome as described in the above section, except that the sequence picker was plasmid pFC91, which carries the untagged $\Delta(zdd355-zdd370)$ deletion (Table 1). Because all rearranged strains carried a *tet* fragment inserted within the *zdd355–zdd370* region (Figs. 3 and 5), the *in vivo* recombinant plasmids could be directly selected for resistance to tetracyclin. Restriction maps of these plasmids were eventually determined. In all cases, they corresponded to the predicted structures of the various rearrangements. The ancestors (before insertion of the Ωdif fragment) of strains, except LN3162 and LN3288, presented in Figure 4B have been verified by this procedure.

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Restriction of the activity of the recombination site dif to a small zone of the Escherichia coli chromosome.

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