Polarization of the *Escherichia coli* chromosome. A view from the terminus

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Abstract — The *E. coli* chromosome replication arms are polarized by motifs such as RRNAGGG oligomers, found preferentially on leading strands. Their skew increases regularly from the origin to *dif* (the site in the center of the terminus where chromosome dimer resolution occurs), to reach a value of 90% near *dif*. Convergent information indicates that polarization in opposite directions from the *dif* region controls tightly the activity of *dif*, probably by orienting mobilization of the terminus at cell division. Another example of polarization is the presence, in the region peripheral to the terminus, of small non-divisible zones whose inversion interferes with spatial separation of sister nucleoids. The two phenomena may contribute to the organization of the Ter macrodomain. © 2001 Société française de biochimie et biologie moléculaire / Éditions scientifiques et médicales Elsevier SAS

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1. Introduction

Two main sites of the circular *Escherichia coli* chromosome are implicated in the cell cycle: *oriC*, where replisomes are assembled for bidirectional replication (for review see [27]), and the diametrically opposite *dif* site, where chromosome dimers, resulting from an odd number of exchanges between sister chromosomes, are resolved [2, 18, 37]. Each of the two *oriC-dif* arms displays a polarization in opposite direction which is imprinted at the sequence level. The replication pause sites (Ter sites) belonging to each arm are all identically oriented, and inhibit forks that move opposite to the natural direction [13]. Chi sites, which control the activity of the RecBCD complex, are preferentially oriented to favor recombination repair of failed replication forks [17]. Genes are preferentially oriented to be transcribed in the same direction as they are replicated [4]. If mutation and selection were the same for the two strands of DNA, long-term evolution should tend toward an equilibrium in which each strand contains an equal number of A and T and of G and C [22]. In *E. coli*, this parity rule holds for the complete genome but not for individual segments, which often display a composition bias for which Lobry coined the term chirochore [23]. When the complete genome sequence was assembled, it appeared that a chirochore structure is found along each replication arm, hence the new term replichore [3]. *E. coli* replichores show a moderate composition bias, since the ratio \((G+C)/\text{A}+\text{T}\) is only +3% on the leading strands, but an important fraction of this excess is found in short motifs distributed with a strand bias which inverts near *oriC* and near *dif* [33]. They belong to two major families, both G-rich on the leading strands. One is characterized by the TGGT motif and includes the Chi site; the other prominent family is a purine-rich motif with RRNAGGG (R = purine, N = any base) as the minimal consensus. They account for about 20% of the G-excess on leading strands. Already clear at the heptamer level, the skew is maximal for the octamer motif RRNAGGGS (S = G or C). The distribution on the chromosome of oriented RRNAGGGG motifs is presented in figure 1. The skew, in general higher than 70% on the leading strand, increases almost monotonously from *oriC* to *dif* to reach a value above 90% in the 400 kb surrounding *dif* (figure 1A). It thus appears that the skew inversion in the terminus is a good indicator for the position of *dif*, whereas the other skew inversion is only a poor indicator of *oriC* position (figure 1B). The two chromosome arms skewed in opposite directions by the RRNAGGGS motifs (for which we suggest to conserve the term replichore) do not coincide exactly with replication arms. Though generally evenly distributed on either strand, the AGGG tetramer itself is found in the 200 kb surrounding *dif* at 70% on the strands running 5’ to 3’ from *oriC* to *dif*. The asymmetry of strand composition must be the result of the superposition of asymmetric selective and/or mutational pressures related directly or indirectly to replication [10]. Oligomeric motifs such as RRNAGGGS are too large to be mere products of an asymmetric mutation pressure. We suspect that such motifs have a functional significance, whether they origi-
nate from an adaptive recycling of an asymmetric mutation pressure or from some other evolution process. We present here indications that the skewed G-rich motifs may have functions in DNA transactions which occur in a processive manner on the chromosome, perhaps replication itself but, more likely, post-replicative events of nucleoid shaping or mobilization.

The existence of a long-range nucleoid organization of each replicochrome, long suspected \[24, 32\], is now supported by an increasing body of evidence. For instance, cytological studies have shown that origin and terminus regions move within the cell with different choreography as nucleoids are replicated and partitioned, suggesting the existence of two macro-domains in the nucleoid, the Ori and Ter domains \[29\]. We wish here to review two phenomena restricted to the Ter region, which indicate the occurrence of functional polarization events in this region. These events which involve the interplay of multiple polar elements are: regional control of \textit{dif} site activity and terminal recombination, and organization into non-divisible zones of the 30% of the chromosome centered on \textit{dif}. Though these analyses were not directly aimed at

**Figure 1.** The asymmetric distribution of RRNAGGCG motifs on the replicochondes. A. The frequency of RRNAGGCG motifs in direct orientation has been computed for every 10% of each oriC-\textit{dif} arm, on the chromosome sequence \[3\]. The data are plotted versus the position on each segment on a map of the chromosome open at oriC and centered on \textit{dif}. The number of occurrences per segment is fairly constant, ranging from 74 to 112. B. The location of individual motifs near oriC and \textit{dif}. Vertical full lines, direct orientation; dotted lines, opposite orientation. The sequence analyzed is the leading strand for replicochrome 1, and the lagging strand for the replicochrome 2. Replicochondes are named as in Blattner et al. \[3\].
Figure 2. The DAZ phenomenon. The strains analyzed here were all deleted for the normal dif site and carried, at the positions indicated on the map of the region, either a single transplaced dif site or a cassette formed by a kanamycin resistance determinant flanked on both sides by dif sites in direct orientation. In the first series of strains, we compared the viability of the transplacement strain to that of an isogenic Δ(dif) strain, using a co-culture assay which is an indirect measurement of the ability to resolve chromosome dimers (percentage abortive division, filled circles). In the second series of strains, we measured the rate of cassette loss (Km loss per cell generation, open squares) which is a global measure of Xer recombination (i.e., not discriminating between exchanges within a single cassette and those between sister cassettes). Details of these experiments can be found in [31].

deciphering nucleoid behavior during the cell cycle, their results provide some insight into the organization of the Ter domain

2. The dif activity zone (DAZ)

A transplaced dif site is inactive for dimer resolution, unless it is located very close to its natural position [5, 19, 21, 39] The DAZ, a small region of less than 30 kb (figure 2), displays the rather unusual feature of self-regeneration after deletion, even if the deletion eliminates a much larger region than the original DAZ. All available evidence indicates that the de novo DAZ is formed at the boundary between replication arms [5, 19]. In particular, an inactive transplaced dif site may become physiologically active when the intervening DNA between the transplacement locus and the natural position of dif is deleted [5].

The consequences of rearrangements of the dif region by inversions have been especially informative. Péralts et al. [31] have constructed many inversions in the region (figure 3). They observed that inversions of segments including dif have no effect on the activity of the site, even when one endpoint is located less than 1 kb from dif and the other endpoint at more than 150 kb. On the contrary, inversions of segments flanking dif may inactivate the site. Inactivation requires that the endpoint closest to dif maps at less than 20 kb on either side of the site. Inversion of several non-overlapping segments may be harmful: inversions of segments as small as 8 kb (for instance zdc330-zdc338 or zdd347-zdd355 in figure 3) is enough to inactivate chromosome dimer resolution. However, inversion of small segments like zdc334-zdc338 has no visible effects, nor does the inversion of a 8 kb segment located just to the left of dif (zdc338-zdc346). These results may be globally explained by assuming that many sequence elements act in a polar and additive fashion to generate the DAZ (DOPE sequence, for DAZ-organizing polar element) This model was strongly supported by the observation that after inversion of segment zda192-dif, a 150 kb segment located entirely to the left of dif and starting from an insertion tagging a dif deletion (figure 3), a de novo DAZ was generated. This DAZ was located between the region left of the inverted segment and this segment, and not at the other endpoint. The transplaced DAZ was necessarily the result of an interplay between sequences from the rearranged replichore, indicating that all required elements may be found in this replichore. DOPEs are interchangeable and their polarity may be inverted.

One important conclusion of the rearrangement experiments was that sequences potentially able to act as DOPEs are found far away the original DAZ region. DOPEs may also be found outside of the terminus region. They exist for instance on phage λ DNA, and are the causes of the phenomenon termed terminal recombination. The fact that homologous recombination events may occur at high frequency in the terminus region was first discovered in bacteria wild-type for Xer recombination, using a test that measures the frequency of excision of λ prophages inserted between direct repeats in the terminus region [25]. It was then observed that terminal recombination was associated with one prophage orientation only, and that this orientation was inverted on either side of dif [6]. Finally, we demonstrated that: i) terminal recombination is not observed in wild-type bacteria when no λ prophage is inserted near dif; ii) terminal recombination is constitutive (i.e., exists independently of the assay system) when Xer recombination is abolished; and iii) the presence near dif of a λ prophage in the orientation causing terminal recombination inactivates Xer recombination at dif [7]. Our explanation of prophage-induced terminal recombination is that the prophage carries enough DOPEs oriented in the same direction to be recognized as one side of a DAZ by the mechanism of DAZ formation. For the moment, our attempts to dissect λ DNA or the dif region itself in order to identify the sequences able to disturb
normal DAZ formation are not conclusive, probably because several DOPEs acting in concert are needed to get an effect.

The terminal recombination phenomenon indicates that the \textit{dif} region is subjected to a high frequency of recombinogenic events when dimer resolution is abolished. Since terminal recombination is RecBC-dependent [6], double strand ends must be generated in the region in these conditions. Xer recombination at \textit{dif} occurs at division time and requires FtsK, a protein anchored in the septum, in addition to the Xer recombinases [37, 38]. These observations, in conjunction with the data presented here, led us to propose that, when Xer recombination cannot resolve a chromosome dimer, the DNA stretching between the sister nucleoids through the division plane belongs always to the \textit{dif} region and that formation of the septum across the two stretches results in frequent sites of entry for RecBCD. The entry sites could result from direct scission by the closing septum; alternatively, replication forks meeting septum-trapped DNA might fold back, extruding the newly made strands which then anneal to form double strand ends [36]. Whatever the torture undergone by the DNA of the \textit{dif} region, guillotine or garrote, the \textit{dif} region is highly prone to homologous recombination when Xer recombination at \textit{dif} is inactivated by \textit{dif} deletion or transplacement, or by \textit{xerC} or \textit{xerD} mutations, or even by \textit{ftsK} inactivation [7].

Our studies of the DAZ and of terminal recombination led us to propose a co-location model which states that: i) DOPEs are involved in a process of mobilization of the terminus DNA, to move it away from the division plane; opposite polarities implies movements in opposite directions on either side of \textit{dif}. This event could be part of the general process of nucleoid positioning before cell division; ii) in the case of a chromosome dimer, DOPE-based terminus mobilization results in a tug-of-war which locates both sister \textit{dif} sites in the immediate vicinity of the septum; iii) this positioning facilitates the action of the septum-anchored FtsK protein in conjunction with Xer recombinases; and iv) the tug-of-war effect is responsible for the directionality of the recombination reaction.

Proposals iii) and iv) have been recently supported by studies of the effect of overproduction of the FtsK protein on the activity of transplaced \textit{dif} sites. Xer recombination

Figure 3. Consequences of inversions near \textit{dif}. The different segments tested in the inversion assay are indicated on this map of the \textit{dif} region by horizontal bars. Open bars, harmless inversion (no effect on \textit{dif} activity in dimer resolution). Filled bars, deleterious inversions disturbing the dimer resolution activity of the \textit{dif} site. The two lower segments are identical (inversion of \textit{zda192-dif}, except for the position of the reinserted \textit{dif} locus. Details of these experiments can be found in [31].
pro-
ciency was assayed by measuring the frequency of excision of a ‘double-
diff cassette’, and dimer resolution was measured by a viability test (see figure 1 and [31]).

The major observation was that FtsK overproduction could strongly increase Xer recombination but had no effect on the efficiency of dimer resolution (figure 4). We suspect that the increased cellular concentration of FtsK facilitates recombination between diff sites carried by the same chromosome, but does not improve the probability of interaction of diff sites belonging to dimer chromosomes. The DOPE-based tug-of-war effect, which thus appears a critical factor controlling the directionality of Xer recombination at dif, does not seem controlled by FtsK, consistent with the already mentioned fact that the regional specificity of terminal recombination is conserved in ftsK mutants [7].

In conclusion, our analyses of the regional constraints operating on the resolution activity of the dif site provide clear and solid support to the existence of two series of polar elements oriented in opposite direction on either side of dif, which are probably the DNA targets for a processive mechanism acting to pull the terminus DNA away from the division plane. At present, the factors interacting with the polar elements are unknown, but the possibility that each replichore is polarized by DOPEs from oriC to dif is clearly open. If this is confirmed, the mechanism acting near dif may act on the whole chromosome, and be a major player in the nucleoid positioning mechanism.

3. The non-divisible zones (NDZs)

One general approach to the role of chromosome polarization is to analyze the consequences of precisely located chromosomal inversions. Some inversions cannot be constructed, even when they are conservative (i.e., maintaining all gene functions present in the parental strain). This was first observed in E. coli by Konrad [15] for the segment between the lac operon and attφ80, a region we further analyze here. Subsequent investigations

**Figure 4.** Overexpression of FtsK and dif activity. A. Levels of Xer recombination measured by the cassette excision assay at three positions of the terminus (dif, zdd370 and zda192) and one external position (lacZ). Measurements are made in three conditions for FtsK: repressed (filled bars), the strain carries the ftsK::cat1 mutation and a repressed plasmid-borne ftsK+ gene; normal (dotted bars), the strain is ftsK+ wild-type; overproduced (open bars), the strain carries the ftsK::cat1 mutation and an induced plasmid-borne ftsK+ gene. Expression of the plasmid-borne ftsK+ gene was controlled by the pBAD/arabinose system. Shown is the percentage of cassette loss per cell generation. B. Dimer resolution viability assay. Three strains, ∆dif (open squares), wild-type (filled squares), and zdd370::dif transplacement strain (33 kb to the right of the natural position), with FtsK normal level (open circles) or with FtsK overproduced (filled circles), were co-cultured for 80 generations with a reference ∆dif strain. The reference ∆dif strain was TcR and the others were TcS. The ratio of TcR to TcS cells was measured at various times of the competition experiment. This ratio decreases as a function of the level of dif proficiency in dimer resolution. Note that FtsK overproduction does not improve viability (i.e., dimer resolution capability) of the transplacement strain zdd370::dif, in spite of the strong concomitant enhancement of Xer recombination at this position (A).
in several laboratories have confirmed that some chromosomal intervals in *Salmonella typhimurium* as well as in *E. coli* cannot be inverted [9, 28, 32, 34, 35]. Intolerance of inversion may have various causes. Examples of true non-invertible segments have been reported. Miesel et al. [28] have observed segments of the *S. typhimurium* chromosome which, although non-invertible by indigenous recombination, could give rise to well-tolerated inversions when the recombining sequences consisted of exogenous DNA introduced by phage-mediated transduction. Thus, obstacles exist which prohibit the indigenous recombination needed for certain inversions. Rebollo et al. [32] have reported a somewhat different situation in *E. coli*, because, in addition to tolerant and non-invertible segments as in *S. typhimurium*, they observed segments whose inversion is feasible but detrimental. Thus, in *E. coli*, chromosomal segments may be either: i) permissive and tolerant to inversion (type T, the inverted state is stable over generations); ii) permissive but refractory (type R, the inverted state is detrimental); or iii) non-permissive (type N, either the inversion is not feasible or so strongly disabling that it is never detected). These authors have also observed that most deleterious inversions have one endpoint located in the 20 to 30% of the *E. coli* chromosome flanking the replication terminus. termed for this reason non-divisible zones (NDZs), these regions also harbor multiple replication pause sites which, when occupied by the Tus protein, are polar inhibitors of replication fork movement [13]. Tus and pause sites appeared in earlier experiments not to be involved in the NDZ phenomenon because inversions ending in the NDZs remained non-permitted or deleterious in *tus*-deleted strains [9]. Though still true for many segments, this conclusion has to be modified since both in *S. typhimurium* and in *E. coli* some N segments become T in Tus conditions (J. Roth, personal communication; [11]).

It thus appears that different types of constraints may dictate the feasibility of inversions or their phenotypic consequences. It has been proposed that the detriment caused by inverted segments of the NDZs could be an alteration of a higher order nucleoid organization involving interactions between ordered sequences [32]. Here, we report new results supporting this view. A further analysis of the region previously named NDZ1 (between 23 and 30 min on the genetic map) has been performed, to determine more precisely the positions of segments whose inversion is detrimental. Inversions between predetermined positions were sought using a genetic system based on modified Tn10 insertions [32]. The results presented in the left part of figure 5 concern T and R segments, whose inversion had certainly been generated, and exclude a number of segments belonging to the N class (a more complete report will be presented elsewhere; [11]). The existence of T segments encompassing R ones, plus the fact that many R segments do not overlap, makes it unlikely that the deleterious effects are due to reorientations of a single locus or to change in direction of replication. Our general interpretation is that NDZ1 is not a continuum but a succession of several smaller NDZs each harboring sequences whose relative orientation is physiologically important. Long T segments might contain entire NDZs, the R segments might split and inactivate one of these NDZs, whereas small T segments might contain no or only few polar determinants.

One remarkable feature of deleterious inversions is that they all cause the same type of detriment: alteration in the shape and distribution of nucleoids, and cell filamentation. Some examples are presented in figure 6, each involving a different, non-overlapping, segment. We suspect that the growth defect is the consequence of a high risk of fatal problems leading to abnormal nucleoid and cell morphology. The abnormal bacteria are probably destined to die, and the apparent generation time of deleterious inversion strains is in consequence increased. The altered nucleoid morphology closely resembles that observed in decatenase *Par* *E. coli* mutants [14], as if physical separation of sister chromosomes carrying certain inversions has a high probability of failure during the cell cycle. This suggests that the NDZs participate in a proper higher-order organization which facilitates spatial separation of sister chromosomes, perhaps by directing decatenase action.

4. Provisional conclusions and perspectives

The two regional phenomena described here, the positional control of *dif* resolution activity and the topographical constraints revealed by NDZs, are among the most direct indications that features of the sequence command *in cis* the behavior of the chromosome during post-replicative events.

It is tempting to correlate the strong skew of the RRNAGGGS motifs near *dif* with the functional bipolarization of both sides of *dif* which controls the formation of the DAZ. The DOPEs are not yet identified, but our analyses indicate that: i) they are numerous and act in an additive fashion; ii) they provide polarity to regions even distant from *dif*; and iii) their skew must change on either side of *dif*. The RRNAGGGS motifs possess exactly the topographical properties expected for DOPEs. In particular, their skew is inverted at *dif*, and is maximum near *dif* (figure 1). The RRNAGGGS motif is also strongly biased on λ DNA, and in the right direction to explain the effects on *dif* activity of a nearby prophage.

RRNAGGGS motifs are skewed from oriC to *dif*, and this raises the question of their possible involvement in the replication process. Direct involvement is unlikely, at least in the terminus region: the RRNAGGGS motifs display their strongest skew on either side of *dif*, but *dif* is not the meeting point for replication forks [26], and the region may normally be replicated in either direction [8]. Indirect involvement is more appealing: the polarized mechanism
acting on either side of dif to pull the DNA of the terminus away from the division plane may also control the movements of the entire chromosome in the cell, including its passage through the replication factory. This factory seems to occupy a fixed position at the center of the cell, where the future septum will separate the products of a round of replication. Replication arms must move through this factory from origin to the terminus during a replication cycle [16, 20]. If this movement is facilitated by a mechanism involving polar elements, one may expect fork velocity in vivo to depend on the direction of replication. We are currently testing this possibility.

In the second example of functional chromosome polarization, the NDZ phenomenon, the nature of elements generating a NDZ must for the moment remain uncertain, since the present data do not allow precise determination of the number and positions of NDZs in the 23 to 30 min region. The succession of seemingly independent and possibly cooperative NDZs in the 10-min region left of dif leads to the proposal that a given NDZ may be initiated and/or terminated at specialized sequence elements. An NDZ might include such initiator/terminator elements, plus polarization factors. Though the analogy with the situation described in the region surrounding dif (about 200 kb to the right of the region analyzed here; figure 5) is striking, polarization of NDZs might not be achieved by the polar elements so far identified either functionally or at the sequence level. In fact, DOPEs are not necessarily deleterious when inverted: the left side (30 kb) of the DAZ is flanked on its left by a long segment (about 120 kb) whose inversion is harmless, although this PT segment harbors DOPE elements able to substitute for those normally controlling dif (figure 5 and [31]).

Other observations may provide additional leads to analysis of the structure and role of NDZs. First, the sequence of the region where NDZs are found reveals the

![Figure 5](image.png)

**Figure 5.** Harmless and deleterious inversion in the 24–34 min region. The upper horizontal line represents the physical map of the chromosome region analyzed. Map units (kb) are from the standard Ecomap7 [1]. The different chromosomal segments submitted to the inversion assay are represented by horizontal bars, whose limits are indicated on the physical map by vertical dotted lines. Open bars, T segments (tolerated inversions). Filled bars, R segments (deleterious inversions). The method for generating inversions (reconstitution of a wild-type Tn10 by homologous recombination between two differently mutated Tn10 inserted in opposite orientations) has been described in [32]. Details of these experiments will be found in [11]. Only inversions to the left of position 1400 kb are considered for the NDZ phenomenon. Inversions to the right of this position are drawn, most of them from figure 3, for better assessment of the respective positions of the regions involved in the DAZ and in the NDZ phenomena.
potential for a high level of curvature and a low degree of helix stability compared with the rest of the chromosome [30]. Whether these sequence properties are involved in NDZ organization will have to be examined, concurrently with skewed sequences. Second, Higgins has recently discovered that the ability of two res sites inserted into the terminus of the Salmonella typhimurium chromosome to recombine with each other depends upon their relative locations, and he has located several barriers hindering communication between the sites (this meeting). Considering the requirements for recombination between res sites, such barriers must behave as elements that prevent supercoiling diffusion [12], making them good candidates for limits between NDZs.

Finally, the Ter macro-domain, recently described by Niki et al. [29] and characterized by a co-location in the cell of all tested segments of the domain during the cell cycle, coincides well with the region where NDZs are found. The behavior of the Ter domain in nucleoid dynamics may depend in part on its organization in NDZs, itself determined by specific sequence elements, and in part on the DOPE-dependent mechanism of mobilization.

Figure 6. Micrographs of bacteria carrying deleterious inversions. Exponentially growing cultures of clones carrying the inversions indicated on figure top (aroA-fadR; fadR-pyrF; pyrF-zdi378) were stained by DAPI and examined by combined phase contrast and fluorescence microscopy. Abnormal bacteria represented a few percent of the populations. Nucleoids appear as red masses (false colors).
Chromosome polarization in \textit{E. coli}

In both cases, future research may lead to the identification of the various sequence elements involved, by combining tests for detecting interactions between distant parts of the chromosome and computational genome analyses, as well as the molecular characterization of other partners involved. One may predict that the 4-D representation of the fate of the terminus region in the course of the cell cycle will be an important chapter in a future biology text: ‘Nucleoid Tectonics’.

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