DNA segregation during the cell cycle of Escherichia coli.
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Chapter 1

Introduction

1.1 Organisation of DNA in the cell

1.1.1 Introduction

In this thesis we address the dynamics of DNA organisation of the prokaryote (and eubacterium) *Escherichia coli* in relation to the cell cycle. During the cell cycle all DNA is duplicated and segregated (partitioned) to each nascent daughter cell. Of course, DNA provides a cell with an information database, which the cell uses to make its constituents. The complete genome sequence of *E. coli* has been available since 1997 (Blattner *et al.*, 1997). Nevertheless, however important DNA may be, living cells exist by virtue of a combination of DNA and many other constituents, such as RNA, phospholipids, sugars, proteins and water. Therefore, our aim as molecular cytologists is to find out how these constituents work together in their natural context: inside the cell. With the experiments described in this thesis we have tried to find a fitting description of how DNA segregates in *E. coli* during the cell cycle (chapters 2, 3, and 4). Our results pertain to global DNA-organisation;
underlying factors that determine this organisation were not assessed directly. Therefore, I use this chapter to introduce factors that play a part in determining global DNA-organisation. In chapter 6 I discuss how these factors might be involved in DNA segregation.

Some aspects of DNA organisation are equal among most species, e.g. the basic structure of the DNA-molecule itself. Many other aspects of DNA organisation differ substantially between species, e.g. *E. coli* has its genes on one circular chromosome whereas humans have their genes on 46 linear chromosomes. Treating all that is known about DNA organisation is beyond the scope of this thesis, but because prokaryotes and eukaryotes are often compared in the literature, I will also introduce some aspects of eukaryotic DNA organisation. Species are classified as eukaryotes if their DNA is contained in a membrane-bound organelle, called the nucleus. Prokaryotes lack a nucleus; instead, their protein-poor DNA is separated from protein-rich cytoplasm by physical forces (Odijk, 1998). This gives rise to the nucleoid as a distinct structure, a structure that can be visualised by phase-contrast microscopy (Mason and Powelson, 1956). I should note that conclusions drawn from a comparison between a prokaryote and eukaryote with respect to DNA organisation, might not be applicable to every prokaryote-eukaryote combination. For instance, as a rule prokaryotic DNA is circular and eukaryotic DNA is linear, but some bacterial species contain linear DNA (e.g. *Borrelia burgdorferie*; see Jumas-Bilak et al., 1998 and references therein) and under some circumstances a eukaryote, such as fission yeast, may contain circular forms of DNA (Nakamura et al., 1998).

### 1.1.2 The basic shape of DNA

DNA can adopt different shapes, but it is generally believed that DNA exists in cells as B-DNA: a double helix of two DNA strands, held together by H-bonds between complementary nucleotides (Bates and Maxwell, 1993a). This shape fits well with the fact that the ideal shape of long molecules is helical (Maritan et al., 2000), but in the case of DNA it imposes an interesting biological problem: because the two strands of the
double helix are twisted around each other they cannot be separated without breaking the double helix, they are linked (fig. 1.1).

Theoretically, this 'linkage problem' occurs only when the ends of the double helix are joined to form a circle. When the two strands of a linear DNA molecule are twisted around each other $Tw$ times, and the ends of this molecule are joined, this 'twist' is constrained within the molecule. Chromosomes and plasmids in most bacteria are circular, but eukaryotic chromosomes are long linear DNA-molecules. However, if DNA is very long, or if the ends of a region of DNA are fixed in some way (by DNA-binding proteins, for instance), the two strands can also be considered 'linked'. Linkage problems are solved by topo-isomerases, which are enzymes that can break and rejoin DNA (see section 1.1.5.7; for an extensive review about topo-isomerases see Wang, 1996; for a more general description see Bates and Maxwell, 1993c).

1.1.3 DNA geometry and DNA topology

Because of the linkage problem, introduced in the previous section, the geometry of a closed-circular DNA-molecule, or a bound region of DNA, can change. Twist ($Tw$) can be converted into 'writhe' ($Wr$), which is the twisting of the double helix around its central axis. This changes the geometry of the DNA molecule, such that it forms higher-order 'supercoils'. Twist and writhe are geometrical parameters. The 'linkage number' $Lk$ is a topological parameter. It represents the number of links in a closed circular DNA molecule. The linkage number is equal to the sum of twist and writhe (Bates and Maxwell, 1993b):

$$Lk = Tw + Wr$$

Thus, whilst the topology of a DNA molecule remains constant, its geometry may change (fig. 1.2).

Supercoiling can have an effect on how 'active' DNA is. On average, the two strands of linear DNA are twisted around each other $360^\circ$ every 10.5 base pairs (Watson and Crick, 1953). This represents the 'relaxed' state of
DNA. Increasing or decreasing $l_k$ may introduce torsional stress into a DNA molecule. Because this stress cannot be released from circular or otherwise bound DNA, it represents a certain amount of free energy (Bates and Maxwell, 1993b). DNA-binding proteins, such as topoisomerases, may use this energy. Proteins that increase torsional stress do this at the expense of energy (e.g. DNA gyrase in bacteria uses ATP to increase negative supercoiling; see Hsieh et al., 1991; van Workum et al., 1996; Westerhoff et al., 1988).

Without DNA-binding proteins, DNA forms plectonemic supercoils (fig. 1.2b); with specific DNA-binding proteins, DNA can form solenoidal supercoils (fig. 1.2c; section 1.1.5.6). Plectonemic supercoiling brings about limited compaction of a DNA molecule (~2.5 fold; Boles et al., 1990). It is the predominant type of supercoiling in bacteria. Solenoidal supercoiling brings about a higher level of compaction. It is the predominant type of supercoiling in eukaryotes (nucleosomes).

In most cells DNA is negatively supercoiled, i.e. under-twisted (see for instance Higgins, 1999). In general, negative supercoiling increases the meltability of DNA. This makes sense, because DNA-related processes often require melting of the double helix (see for instance section 1.1.5.2). Environmental conditions may change the average state of supercoiling (e.g. Hsieh et al., 1991), and, therewith, global gene expression.

Despite general believe, it is possible to construct double-stranded DNA in such a way that it seems supercoiled without being linked. Due to steric hindrance, a single-stranded DNA-molecule cannot rotate completely freely around its axis (Wu and Wu, 1996). Therefore, if a single-stranded DNA-molecule is twisted on itself and its ends are joined to form a single-stranded circle, it may not resume an open circular form but may remain twisted (Wu and Wu, 1996). If two complementary strands are twisted in this way and rejoined by H-bonding, then the resulting molecule will look very similar to a linked supercoiled double-helix. However, the strands are not linked and can be easily separated. There is some experimental data that supports this model of supercoiling
that compaction of replicated DNA, after leaving the replisome and mediated by MukB, could lead to DNA segregation (Dasgupta et al., 2000; Graumann, 2001; Holmes and Cozzarelli, 2000 and references therein; see also section 6.1.2.4).

Immuno-labelling of MukB provided direct evidence for a role in organising DNA in *E. coli* (Den Blaauwen, submitted). An estimated number of 150 MukB molecules per cell (Kido *et al.*, 1996) produced distinct foci that co-localised with nucleoids during all of the cell cycle (fig. 1.6a). A role in organising DNA is further supported by the structure and size of MukB (fig. 1.6b). It has the distinctive five-domain structure of an SMC protein: an N-terminal globular domain containing an ATP-binding site, a long coiled-coil, a hinge, another long coiled-coil, and a C-terminal globular domain containing a DNA binding site (Melby *et al.*, 1998; Niki *et al.*, 1992). Most eukaryotic SMC proteins are present as hetero-dimers, probably with their coiled coils in anti-parallel conformation. MukB and most bacterial SMC proteins form anti-parallel
homo-dimers (Melby et al., 1998). An anti-parallel dimer should be able to bind DNA at both ends. As all SMC proteins, MukB is huge (170 kDa; Niki et al., 1992). The coils are around 65 nm long and the hinge is flexible: the angle between two arms may range from 1° to 180° and possibly beyond (Melby et al., 1998). MukB should be able to operate on two strands of DNA 100 nm apart. This is a large distance compared to the size of E. coli (fig. 1.6c).

1.1.5.5 Other nucleoid-associated proteins
Although SMC proteins are now considered to play an important role in structuring the bacterial genome, about ten other DNA-binding proteins are traditionally associated with nucleoid structure (Azam and Ishihama, 1999). I will introduce the most abundant ones briefly (for a review see Pettijohn, 1996).

The five most abundant nucleoid-associated proteins in E. coli growing exponentially in rich medium are (in order of abundance): Fis, Hfq, HU, and the closely homologous proteins StpA and H-NS (Ali Azam et al., 1999). Their relative quantities change with growth phase (Ali Azam et al., 1999). In stationary phase, the level of Fis drops below detectable levels, whilst the proteins Dps and IHF, present at low levels in exponential phase, become the first and second most abundant proteins. The order of DNA binding affinity was determined to be: HU \( (K_d = 25 \text{ nM}) \), Hfq, Lrp, CpbB, Fis, H-NS, StpA, CpbA, IciA, and Hfq/Dps \( (K_d = 250 \text{ nM}) \) (Ali Azam et al., 1999).

Fis is a small DNA-binding protein that probably influences DNA topology by regulation of topo-isomerase activity and by modulating super helical density (Schneider et al., 1999; Schneider et al., 1997). Hfq (host factor for integration of phage Q) binds both DNA and RNA (Kajitani et al., 1994). It binds preferentially to curved DNA, independent of the underlying nucleotide sequence (Ali Azam et al., 1999). HU was initially called 'histone-like', because SV40 DNA together with HU and a 'nicking-closing' enzyme produced histone-like beads under the electron
1.1.5.6 Histone-like proteins and true histones

Histone-like proteins and true histones

Nucleoid-associated proteins in prokaryotes are often coined 'histone-like' proteins (previous section and Schmid, 1990), because they are basic, small, abundant, and bind DNA in a sequence-independent manner. However, proteins capable of organising DNA as stable solenoids (fig. 1.2c), a defining feature of true histones, have only been found in eukaryotes and archaea-bacteria, but not in eubacteria (Li et al., 1999; Sandman and Reeve, 2000).
In eukaryotes, DNA is wrapped around complexes of eight histones, forming beads called nucleosomes. Nucleosomes constrain negative supercoiling, which protects DNA from ligands. When DNA is released from the histone-complex the negative supercoiling promotes unwinding (Bates and Maxwell, 1993d and references therein), which makes the DNA more susceptible to ligands. A fibre consisting of nucleosomes has a diameter of 10 nm. This '10 nm fibre' is further condensed, first in a 30 nm fibre, then into 'chromonema', which are fibres with a diameter of around 100 nm; in metaphase chromosomes DNA is further packed into 200 nm and 400 nm fibres (Visser, 1999 and references therein). During interphase, DNA is present in various degrees of compaction. The most compact form of DNA folding is reached in metaphase, although the average difference between the degree of compaction of DNA between interphase and metaphase is probably limited (see for instance Manders et al., 1999). In HeLa cells, the amount of de-compaction of chromatin from anaphase to G1 was quantified from 3-D time-lapse images of GFP-tagged histones. All chromatin de-condensed by at least a factor of 3 (the average factor was 5), and no substantial re-arrangement of chromatin domains took place (Manders et al., in preparation).

1.1.5.7 Topo-isomerases
Topo-isomerases have already been mentioned in the previous text, because of their role in DNA supercoiling. They change the linking number of DNA, hence, its topology, by transiently cutting DNA (Bates and Maxwell, 1993c; Wang, 1996 and references therein). Two types of topo-isomerases have been defined: type I topo-isomerases cut one strand of the double-helix to allow one strand to pass the other; type II topo-isomerases make a double-strand break to allow one helix to pass another (e.g. Brown and Cozzarelli, 1979).

Most topo-isomerases use the free energy contained in supercoiled DNA. An exception is DNA-gyrase, a type II topo-isomerase found only in prokaryotes. Although the enzyme is capable of relaxing negatively supercoiled DNA by increasing the linking number, it can also introduce
negative supercoiling in relaxed DNA by reducing the linking number of DNA (Gellert et al., 1976). Because the latter is energetically unfavourable, it does this at the expense of ATP. Thus, in prokaryotes DNA-gyrase keeps DNA under negative torsional stress. In eukaryotes, no such topo-isomerases have been found. They may be obsolete in eukaryotes, because the combined action of DNA-organising proteins, such as histones and relaxing topo-isomerase, can also produce negative supercoiling upon protein dissociation. Chromatin remodelling in eukaryotes is achieved by ATP-dependent remodelling-enzymes, which are part of multi-subunit complexes (Peterson, 2000). Remarkably, bacterial DNA is wrapped, be it transiently, around DNA-gyrase reminiscent of the way DNA is wrapped around the histone octamere in eukaryotic nucleosomes (Bates and Maxwell, 1993c).

Even though DNA gyrase is an essential enzyme, its 'control' over supercoiling in E. coli is limited. Jensen and co-workers were able to modulate the expression level of DNA gyrase and quantified its amount of control as the percentage change in the amount of supercoiling in response to a 1% change in the level of DNA-gyrase expression (Jensen et al., 1999). They found that the amount of control was only 0.2 % when expression of DNA gyrase was modulated around wild-type levels (see Jensen et al., 1999 for details). Apparently, DNA supercoiling is subtly controlled in E. coli.

1.2 DNA organisation and the cell cycle

The organisation of DNA in a living cell is not static in time. Many processes influence DNA organisation at different times during the cell cycle. One of the more prominent ones is DNA replication. Thus, to understand intracellular DNA-organisation in E. coli, we need to know the timing of DNA-replication within the cell cycle.

1.2.1 Timing of replication within the cell cycle of E. coli

The relationship between the replication cycle and the cell-division cycle
Figure 1.7 - Cell cycles in different strains of *E. coli* and under different growth conditions

- $T_d$: cell doubling time; $C$: time to replicate one chromosome equivalent; $D$: time from termination of replication to cell division; $I$: initiation time. Every $I+C+D$ minutes a new $I+C+D$ cycle starts (note that $I=T_d$ under steady state conditions).

Top: cell cycle of *E. coli* strain K-12, derivative MC4100, grown steady state in minimal glucose medium at 28°C (conform the experiments described in this thesis; Huls *et al.*, 1999).

Middle: "classic" cell cycle parameters in which $C$ and $D$ are more or less constant over a range of growth rates (measured in B/r strains);

Bottom: multi-fork replication at a fast growth rate in the same B/r strain.
of *E. coli* can be described using the 'I+C+D rule' (fig. 1.7; Helmstetter, 1996). *I* is the time required to achieve the capacity for initiation of replication, which is defined by the initiation mass of a cell; *C* is the time required to replicate one chromosome; *D* is the time in between termination of replication and cell division. Thus, when the initiation mass is reached, in *I* minutes, it takes *C*+*D* minutes from initiation of replication, via termination of replication, to cell division. Under steady-state conditions growth conditions are constant by definition. Consequently, *I*, *C*, and *D* remain constant: an *I*+*C*+*D* cycle starts every *I* minutes, and *I* equals the doubling time *T*ₜ. When *C*+*D* ≥ *T*ₜ then initiation of replication precedes cell birth. Thus, a cell is born with at least two origins, because the replication cycle is already underway. When *T*ₜ < *C*, then a new round of replication initiates before the previous one has terminated (multifork replication; fig. 1.7c). When *C*+*D* ≤ *T*ₜ, then a single round of replication initiates *T*ₜ−(*C*+*D*)=*B* minutes after cell birth, in which *B* is the time between cell birth and initiation of replication.

Cell-cycle parameters depend on growth conditions and on the type of strain (Bipatnath *et al.*, 1998; Helmstetter, 1996). To illustrate, figure 1.7 schematically depicts three cases: the first represents the K-12 strain used in our experiments, growing with a doubling time of *T*ₜ=80 min. (cf. chapters 2 and 3; Huls *et al.*, 1999); the second represents a different strain, growing with the same doubling time (*T*ₜ=80 min.), but with different values for *C* and *D* (cf. Cooper and Helmstetter, 1968); the third also represents this strain, but now growing with a different doubling time (*T*ₜ=20 min.) whilst *C* and *D* remain constant (cf. Cooper and Helmstetter, 1968). The third case exemplifies multi-fork replication.

Helmstetter summarised cell-cycle parameters of three *E. coli* B/r strains and several *E. coli* K-12 strains grown at different growth rates (Table 1 in Helmstetter, 1996). In this list the cell cycle parameters of K-12 strains show a lot of variability, probably because the various K-12 strains are genetically and physiologically different. In the list of Helmstetter, B/r strains appear to have a more or less constant *C* and *D* period at growth
rates below 60 minutes, but more recently Bipatnath and co-workers have shown that C may decrease gradually from C=70 minutes at a doubling time of 100 minutes to C=33 minutes at a doubling time of 20 minutes in both a K-12 and a B/r strain (Bipatnath et al., 1998). Because of these uncertainties, we chose our strain and growth conditions such that we could use experimentally determined values for C, D, and T_d (chapters 2 and 3).

1.2.2 Differences in the timing of DNA replication and DNA segregation between prokaryotes and eukaryotes

With respect to the timing of replication within the cell cycle, prokaryotes and eukaryotes are distinctly different. In *E. coli*, for instance, DNA replication coincides with DNA segregation, and sister copies are segregated completely, whilst in eukaryotes, replication coincides with the alignment and cohesion of sister chromatids. The chromatids are separated later in the cell cycle, during mitosis (fig. 1.8). Moreover, bacteria have the ability to initiate a new round of replication before previous rounds of replication have finished. In fast growing bacteria, many 'generations' of replication cycles may take place simultaneously. For instance, a fast-growing bacterium could contain 16 or more copies of the origin of replication, even though there is only one origin per chromosome. Eukaryotes replicate all their DNA once and only once during S-phase. Despite the differences, some researchers propose that the mechanism behind DNA segregation in bacteria may be compared to the mechanism underlying mitosis in eukaryotes (e.g. Begg and Donachie, 1991; Lin et al., 1997; Moller-Jensen et al., 2000; Niki and Hiraga, 1998; Sharpe and Errington, 1999; Webb et al., 1997; Wheeler and Shapiro, 1997). I feel, however, that it may be more useful to compare the initial separation of nascent DNA in prokaryotes to that in eukaryotes, thus during S-phase (see section 6.2.3).
1.2.2 Differences between prokaryotes and eukaryotes

Figure 1.8 - Cell cycles of *E. coli* K-12 and a typical eukaryote

Top: cell cycle of *E. coli* strain K-12 grown in minimal glucose medium at 28°C (see figure 1.7). Bottom: 'typical' eukaryotic cell cycle. For simplicity only one chromosome is depicted. G1: (gap) phase following cell division, chromosomes consist of one chromatid; S: phase in which all DNA is replicated (synthesised) exactly once; G2: (gap) phase preceding mitosis, chromosomes consist of two chromatids held together by cohesins. Mitosis is divided into four phases: P: during prophase chromosomes condense maximally; M: during metaphase chromosomes align in the metaphase plate in the middle of the cell by pulling and pushing of microtubules; A: during anaphase chromatids are separated and segregated to opposite cell poles by microtubules; T: during telophase chromosomes decondense before cell division.

Note the difference in time scale between pro- and eukaryotes. Also note that in prokaryotes DNA replication and DNA segregation are not separated in time as in eukaryotes (S-phase and mitosis respectively).
1.3 Studying the organisation of DNA in *E. coli*

The size of many of the structures introduced in this chapter, are below the resolution of a light microscope (~250 nm; see chapter 3). Nucleoids appear as large, somewhat lobular, structures that segregate gradually in line with cell elongation (van Helvoort and Woldringh, 1994). Two methods are currently used that enable the study of sub-nucleoid DNA regions using fluorescence light-microscopy.

1.3.1 FISH

Fluorescent *in situ* hybridisation (FISH) relies on hybridising a DNA-region of interest with a complementary DNA probe. The DNA of the probe is conjugated with a fluorescent dye or with a molecule that can be detected by fluorescently labelled antibodies. FISH is not a gentle method, it requires cells to be fixed and made permeable to the DNA probe, and the samples need to be heated to about 80 °C in the presence of formamide or incubated in 0.1 M NaOH in order to 'melt' the DNA, which is needed for hybridisation with probe DNA. On the other hand, cells can be cultured under steady-state conditions, which is a prerequisite for reconstructing DNA-organisation as a function of the cell cycle.

1.3.2 GFP

The second method uses green-fluorescent protein (GFP) to tag a DNA region (Margolin, 2000; Robinett *et al.*, 1996). GFP is fused to a DNA-binding protein that binds specifically to a DNA region inserted at a specific position in the genome. For instance, we describe in chapter 4 preliminary results of a time-lapse experiment with GFP fused to LacI, which binds to a cassette of *lacO* sequences inserted near the origin of replication of the *E. coli* chromosome. The same system had been used earlier in *B. subtilis* (Webb *et al.*, 1998) and in *E. coli* (Gordon *et al.*, 1997). The big advantage of this method is that it can be used to monitor the dynamics of DNA in living cells. However, for time-lapse microscopy cells need to be on a slide under the microscope. These are non-ideal
circumstances and certainly not 'steady-state'. Moreover, the amount of GFP-fusion protein is difficult to control. The system that was used in these cases can lead to heterogeneity with respect to the amount of GFP per cell, and possibly to artificial clustering of an excess of fusion protein (forming inclusion bodies; see section 5.2.3). In summary, FISH is the method of choice if information about average movement of DNA as a function of the cell cycle is required. GFP studies are better suited for studies on DNA dynamics, although one should be cautious about extrapolating to a (steady-state) population.

1.4 Outline of this thesis

1.4.1 Outline in brief

In this thesis, an analysis of DNA segregation in *E. coli* as a function of the cell cycle is described. We have studied the average intracellular position of three regions of the *E. coli* chromosome by FISH (chapters 2 and 3), and used computer simulation to test if three particular models explain our FISH data sufficiently. Because we found that these models did not fully explain our data, I re-examine the assumptions on which they were based and propose a new model, based on diffusion of DNA (chapter 6). Finally, after having introduced factors that have an influence on DNA structure in this chapter, I discuss in chapter 6 how some of these factors may be involved in DNA segregation, and take a brief look at how we can make a model incorporating such factors. In the same chapter I give my view on comparisons that are made between DNA segregation in bacteria and in eukaryotes.

1.4.2 Chapter 2

In chapter 2 the position of the origin of replication as a function of cell length is described. Regression analysis was performed on sub-sets of data (distances from a cell pole) to find average positions of oriC as a function of cell length. Previously measured values of C and D were used
to select these sub-sets from the total data set. We concluded that the average distance from a cell pole to oriC remains constant, which is consistent with a model for gradual separation along with nucleoid segregation.

1.4.3 Chapter 3

In chapter 3 we extended the analysis of chapter 2 with two more regions on the E. coli chromosome. In this case our regression analysis incorporated all data-points. We further emphasised that when doing FISH we should always consider the possibility that not all DNA regions in a cell are labelled. This led us to propose a model that describes average positions of DNA regions as a function of cell length.

1.4.4 Chapter 4

In chapter 4 models based on interpretations of FISH data and GFP data were evaluated by computer simulation. We simulated FISH data by computer and compared the results with the experimental data. The results of the 'random model' came closest to the experimental results, probably because the amount of variation in the experimental data was large; a large amount of variation is an intrinsic property of the random model. We argued that the three studied DNA regions (oriC, ftsQAZ, and minB) were not positioned completely random, because the measured distributions of these regions were different. The random model would not predict this. We concluded that movement of a DNA-region might be the result of various factors. We proposed that one of these factors could be diffusion of DNA within a confined region (cf. Marshall et al., 1997).

1.4.5 Chapter 5

In chapter 5 I summarise the results of our FISH experiments, and carefully (re-) examine assumptions on which the models that we tested in chapter 4 were based. Incorrectness of these assumptions may explain some of the variation in our FISH data, and possibly why the distribution
of the simulated data-points differed from the measured distribution.

1.4.6 Chapter 6

In chapter 6 I take another look at some of the factors that influence DNA structure in *E. coli*. In particular, I discuss how they might be involved in a mechanism that segregates replicated DNA. Because such a mechanism is probably based on non-linear interactions between many factors, it is difficult to define a proper model, but it can be even more difficult to predict what kind of data this model would produce. Nevertheless, I discuss some methods that could get us closer to finding a model that produces data similar to our FISH data. I also discuss how a comparison between prokaryotes and eukaryotes could help us to find the mechanism behind DNA segregation in bacteria. Unfortunately, I have to conclude that the mechanisms involved are probably fundamentally different. Finally, I conclude that a new model of DNA segregation in *E. coli* could be based on diffusion of DNA.

1.5 References


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