Investigation of E. coli genome complexity by means of fluorescent reporters of gene expression

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Investigation of *E. coli* genome complexity by means of fluorescent reporters of gene expression.

Doctoral thesis of

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Statutory Declaration

I, **ELISA BRAMBILLA** , hereby declare that I have written this PhD thesis independently, unless where clearly stated otherwise. I have used only the sources, the data and the support that I have clearly mentioned. This PhD thesis has been submitted for conferral of degree at Université Pierre et Marie Curie (Paris), as stated in the joint supervision agreement between Jacobs University and UPMC.

Cachan, January 4, 2015

Signature _____

Abstract

Escherichia coli is a highly adaptable organism, able to survive in many different environments. The information necessary for this adaptation is encoded in the chromosome. The *E. coli* chromosome is a circular molecule condensed in a compact DNA-protein structure, called the nucleoid. The nucleoid is dynamically organized at different levels by DNA supercoiling, the binding of proteins and by the DNA replication and gene transcription processes. The chromosome is not uniform, and shows uneven distributions of nucleoid-associated proteins (NAPs) binding sites, AT-rich sequences and general protein occupancy domains. All these factors can affect gene expression. It has been demonstrated that the position of important genes is highly conserved in γ-Proteobacteria, with genes involved in exponential growth found closer to the origin than genes important during adaptation to stationary phase. These differences along the chromosome and the fact that the position for important genes is conserved lead to the idea that the position of the gene can influence gene expression, and that we can use gene expression to monitor the characteristics of the chromosome. Following this idea, I employed insertions of a fluorescent reporter gene in six different positions around the chromosome. The fluorescent reporter expression is under the control of the promoter of the *hns* gene encoding H-NS, an abundant NAP involved in chromosome shaping, global transcriptional silencing and in several stress responses. For each of these chromosomal positions I measured promoter activity in the different growth phases as a function of growth rate. These results show that in all the positions there is a decrease of protein concentration at fast growth rate, and at the transition into stationary phase. For one of the positions the level of gene expression is significantly lower than in the others, and there is a weaker growth rate dependence of protein concentration. This reduced expression is more evident at lower temperature. This temperature-dependent behavior is consistent with a more efficient repression by H-NS in this position. We propose that this position is different with respect to the others because it is located in a region with a higher AT-content than the genome average. A similar effect of a stronger H-NS repression is visible also for another position near the origin of replication in specific conditions such as at slow growth, low temperature and at entry into stationary phase. These results therefore show that the level of H-NS repression depends on a local position effect and on the growth condition. In order to derive further information about chromosome organization, I also inserted in the chromosome differently regulated promoters such as the ribosomal promoter *rrnBP1* and the T5 phage P5 promoter. The *rrnBP1* promoter is sensitive to supercoiling, while P5 is a constitutive (i.e.

non regulated) promoter. I measured the expression of these reporter strains under different growth conditions and found that an increase in growth rate affects the two promoters differently, with *rrnBP1* expression increasing faster than P5, as expected since an increase in growth rate implies an increase in the DNA supercoiling level and a decrease in ppGpp. I also studied the impact of an inhibition in translation efficiency on the expression of *rrnBP1* and P5 by using sub-lethal concentrations of chloramphenicol. Interestingly, both promoters are induced in presence of the antibiotic, while in the literature an up-regulation effect has been previously reported only for ribosomal genes. Finally, I measured the expression of both promoters in the presence or in the absence of a divergent neighboring gene. I showed that the effect of the neighbor gene depends on the chromosomal position, providing support for the existence of different levels of supercoiling in the *E. coli* chromosome. Our approach therefore allows us to measure how chromosomal properties (such as supercoiling or protein occupancy) can depend on the chromosomal coordinate as a function of different growth conditions, and how the chromosomal position can thus affect gene expression.

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1 Who is *E. coli*?

Escherichia coli, one of the best-studied microorganisms in the laboratory, remains an important model organism for genetic studies. The primary niche occupied by *E. coli* is the lower intestinal tract of mammals, where it resides as a beneficial component of the commensal microbiota. Beside this niche, *E. coli* is able to survive and to adapt to a variety of changes in environmental conditions, such as fluctuations in temperature (Bennett and Lenski, 2007; Riehle et al., 2003), pH (Hughes et al., 2007), UV irradiation (Alcantara-Diaz et al., 2004) and the presence of high ethanol concentration (Goodarzi et al., 2010). In addition to this evolutionary adaptation, *E. coli* can counteract abrupt shocks by activating different pathways and stress responses, such as the glutamate-dependent acid resistance (GDAR) (Castanié-Cornet et al., 2010), or the cold shock response (Jones and Inouye, 1994). The response of the *E. coli* cell to external conditions involves changes in growth rate and cellular physiology. In order to adapt to environmental changes, *E. coli* needs to modify several cellular parameters, such as DNA, rRNA per cell and macromolecular, lipid and protein composition (Schaechter et al., 1958) including chromosomal proteins (Ali Azam et al., 1999). The key for organizing these changes in a coordinated way is in the interplay between Nucleoid-Associated Proteins (NAPs) and DNA conformation via the level of DNA supercoiling (Balke and Gralla, 1987; Hsieh et al., 1991a) resulting in the change in expression of large sets of genes (Travers and Muskhelishvili, 2005a). In the first part of the introduction I will describe the organization of *E. coli* chromosome and the role of NAPs in gene regulation; I will then focus on the cellular adaptation to different growth conditions.

1.1 The *E. coli* chromosome: a dynamically organized structure

The *E. coli* genome is a circular molecule of 4.6Mb that needs to be highly compacted in order to fit inside the cell (Holmes and Cozzarelli, 2000) via a nucleo-protein structure called 'nucleoid' (Valkenburg and Woldringh, 1984). This compaction needs to be tightly regulated in order to have an accurate DNA replication and segregation and cell division (Draper and Gober, 2002), as well as in order to maintain the genes accessible to RNA polymerase. The spatial organization of the chromosome is due to its structural characteristics such as the presence of loop domains and of larger

scale macrodomains that depend dynamically on several factors including DNA supercoiling and NAPs binding.

A supercoiled domain has been defined as the portion of DNA that becomes relaxed when a single or double-strand break occurs (Postow et al., 2004). In the absence of confined supercoiled domains, a single break would be sufficient to relax the entire genomic DNA, leading to the death of the cell (Gellert et al., 1976a). On the contrary, it was shown that only with multiple breaks it was possible to relax the chromosome *in vitro*. The number of breaks required for relaxation (and therefore the number of supercoil domains) was first estimated to be between 6 and 40 nicks per DNA strand (Worcel and Burgi, 1972), and later up to 400, resulting in an average domain size of 10kb (Postow et al., 2004). Higgins et al. found that these domains were variable from cell to cell, were changing over time and were more abundant in exponential than in stationary phase (Higgins et al., 1996). Chromosome organization is therefore very dynamic. Transcription affects the supercoil domains organization: for example it was shown that the activation of a strong promoter can lead to the formation of a new domain boundary in *Salmonella typhimurium* (Scheirer and Higgins, 2001). In addition, it was shown that several proteins are involved in the process of forming and maintaining supercoiled domains. The number of supercoiled domain boundaries is affected by mutations in the gyrase and topo IV enzymes (Staczek and Higgins, 1998). Beside gyrase and topo IV, abundant NAPs such as FIS, HU and H-NS have been shown to stabilize supercoiled loops (Berger et al., 2010; Dame et al., 2000; Schneider et al., 2001). For instance, it was found that the average distance between two FIS binding sites is approximately half the average size of a supercoiling domain, pointing to the structural importance of the presence of two FIS binding regions per domain (Cho et al., 2008).

At a higher level, the *E. coli* genome is organized in several macrodomains. At first, FISH analyses revealed the existence of two macrodomains, the Ori and Ter macrodomains, defined as large regions that localize precisely in the cell, respectively toward the pole and at mid-cell (Niki et al., 2000). Valens et al. studied DNA organization via the percentage of recombination between pairs of sites in the chromosome, and observed the existence of DNA regions which do not interact one with the other (Valens et al., 2004). The results indicated an organization of the chromosome in four macrodomains and two less-structured regions (Figure 1). These regions also show a different mobility within the cell, higher in the non-structured regions and lower in other macrodomains (Espeli et al., 2008).

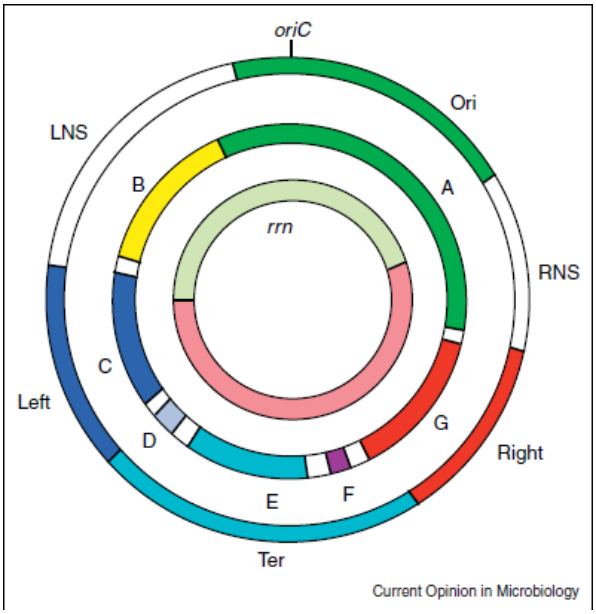


Figure 1 Figure from (Rimsky and Travers, 2011) describing *E. coli* chromosome organization in macrodomains as it was shown by Valens et al. (exterior circle), by Mathelier and Carbone (mid circle), and by Berger et al. (inner circle). The boundaries of these macrodomains appear at similar locations in the chromosome.

With an *in silico* analysis of codon usage, Mathelier and Carbone found a periodical distribution of genes that were divided in two classes, one with metabolic genes and the other with genes involved in cellular processing and signaling (Mathelier and Carbone, 2010). They therefore suggested the existence of functional domains (Figure 1). Berger et al. showed that in a mutant lacking the NAP HU, there was a re-arrangement of upregulated genes in the region of OriC and ribosomal RNA operons with respect to the wild-type strain, pointing to the existence of two distinct structural and functional domains (Berger et al., 2010). Finally, it was discovered that some distal DNA sequences can sometimes be found spatially close together, and in several cases this proximity is related to transcriptional interaction between the sequences (Sexton et al., 2009). All these findings suggest that the organization of the *E. coli* genome in macrodomains corresponds to specific structural and functional needs.

Different proteins preferentially bind to specific macrodomains and are involved in maintaining their structure: for instance, matP targets a specific 13-bp sequence which occurs several times near the terminus, reducing the mobility of the ter macrodomain (Thiel et al., 2012). In addition to this protein, SeqA binds preferentially near the origin and it is excluded from the terminus (Fossum et al., 2007; Sánchez-Romero et al., 2010) while H-NS is often bound to regions correlated with the macrodomain boundaries where pseudogenes and horizontally transferred genes are also found (Zarei et al., 2013).

Finally, RNAP has been shown to be organized in foci at fast growth, therefore condensing the nucleoid (Cabrera and Jin, 2003). The different scales of chromosomal organization are summarized in Figure 2.

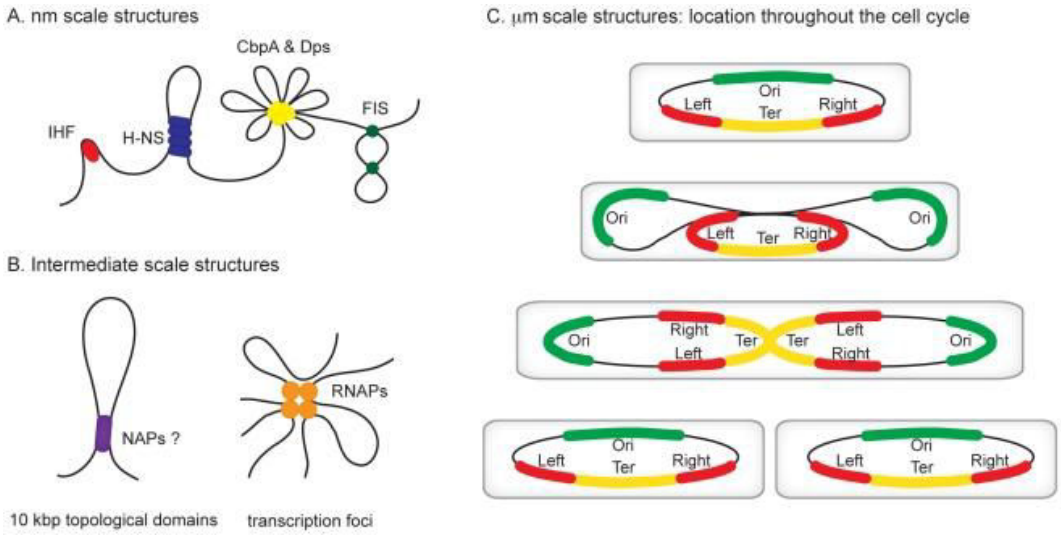


Figure 2 Figure from (Dame et al., 2011) describing the chromosome organization from the nm scale, with proteins interacting with DNA, to an intermediate scale, with NAPs stabilizing loops and plectonemes and RNAP forming transcription foci, to the µm scale, with the chromosome organized in the macrodomains.

Genome supercoiling is a global property that acts both in the spatial organization of the chromosome and in the regulation of gene expression (Gellert, 1981). Negative supercoiling favors the unwinding of DNA, and consequently all the processes connected with helix opening (Vologodskii and Cozzarelli, 1994), such as DNA replication initiation (Marians et al., 1986) and gene transcription (Chen et al., 1994; Geertz et al., 2011). Similarly, activation of transcription is accompanied by an increase in the local level of negative supercoiling by the translocation of the RNAP on the DNA (Deng et al., 2005; Figueroa and Bossi, 1988). It was shown that even a small reduction of the level of negative supercoiling in bacteria can be lethal (Zechiedrich et al., 1997). Superhelical density varies depending on the growth stage of the cell (Balke and Gralla, 1987), its growth rate and the environment (Hsieh et al., 1991a, 1991b). This tuning of the supercoiling level depends on the interplay between DNA gyrase (Gellert et al., 1976b), introducing negative supercoils, and TopoI (DiNardo et al., 1982; Menzel and Gellert, 1983) and Topo IV (Zechiedrich et al., 2000), removing them (see Box 1). Due to the different distribution of gyrase binding sites in the chromosome, specifically more abundant towards the origin (Condemine and Smith, 1990; Jeong et al., 2004), the existence of a gradient in supercoiling in *E. coli* chromosome has been proposed (Sobetzko et al., 2012). However, no direct proof of this gradient has

been found: the comparison of the expression of *gyrA* (activated by a decrease in negative supercoiling) and *lac* (activated by an increase in negative supercoiling) promoters inserted in different chromosomal position did not show significant differences in supercoiling levels along the chromosome (Miller and Simons, 1993). Similar results were found in *Salmonella thyphimurium* with the supercoiling-sensitive *proU* promoter (Pavitt and Higgins, 1993) and more recently by monitoring resolvase activity in different chromosomal positions (Rovinskiy et al., 2012).

Box 1 Gyrase and supercoiling

The level of chromosomal supercoiling in the cell is regulated by the concerted activity of four topoisomerases: gyrase, topo I, topo III and topo IV (Zechiedrich et al., 2000). They all change the level of supercoiling by transiently breaking and re-arranging DNA strands (Nöllmann et al., 2007). Gyrase is the only topoisomerase increasing the level of negative supercoiling (Gellert et al., 1976b) according to the level of ATP in the cell (Cozzarelli, 1980; Cullis et al., 1992). The control of global supercoiling is necessary for efficient DNA replication and for transcription. Both DNA replication and transcription involve the unwinding of DNA double strands, and this introduces positive supercoils downstream and negative supercoils upstream of the ongoing process (Liu and Wang, 1987; Postow et al., 2001). Gyrase activity is necessary in order to remove the positive supercoiling in excess and to allow the replication and transcription progression (Filutowicz and Jonczyk, 1981; Kreuzer and Cozzarelli, 1979). A decrease in negative supercoiling stimulates the expression of the two gyrase subunits gyrA and gyrB (Menzel and Gellert, 1983), and it decreases topoI expression (Tse-Dinh, 1985).

The level of negative supercoiling and the chromosomal structure are dependent on the action of several NAPs (Hardy and Cozzarelli, 2005). NAPs are high abundant proteins that regulate gene expression and shape the chromosome (Maurer et al., 2009). In 1987 Drlica and Rouviere-Yaniv described several histone-like proteins in bacteria (Drlica and Rouviere-Yaniv, 1987), proteins that were able to interact and re-shape DNA. Among these are HU, IHF, FIS, H-NS. HU influences the expression of many genes in *E. coli*, including those involved in central metabolism and the acid stress response (Oberto et al., 2009). It was shown to interact with Topo I, altering as a consequence the superhelicity of DNA (Broyles and Pettijohn, 1986; Malik et al., 1996). IHF is implicated in transcription regulation and DNA replication (Freundlich et al., 1992) and it is able to bend DNA more than 140° (Rice et al., 1996). FIS activates transcription of rRNA operons (Finkel and Johnson, 1992) and re-shapes DNA by increasing DNA branching (Schneider et al., 2001). H-NS is a global repressor

(Rimsky, 2004) that can form prominent foci within the nucleoid (Wang et al., 2011), induces condensation on chromosomal DNA (Dame et al., 2000) and compacts the chromosome upon entry into stationary phase (Thacker et al., 2014). Another NAP, Dps, has been shown to intervene in the nucleoid compaction at entry into late stationary phase (Frenkiel-Krispin et al., 2004). Genome-wide DNA-binding profiles of FIS, HNS, IHF show that the binding of these proteins is more abundant in non-coding parts of the genome (Grainger et al., 2006), supporting the idea that NAPs contribute to the formation of the boundaries between supercoil domains (Zarei et al., 2013). The growth phase dependence of these proteins (Ali Azam et al., 1999) is summarized in Figure 3. The coupling of chromosomal topological state and gene expression in order to adapt to the growth phase also relies on the change in composition of RNA polymerase (Geertz et al., 2011), with the amount of σ^S subunit increasing from undetectable level in exponential phase to 30% of the level of σ^{70} in the transition to stationary phase (Jishage and Ishihama, 1995; Jishage et al., 1996).

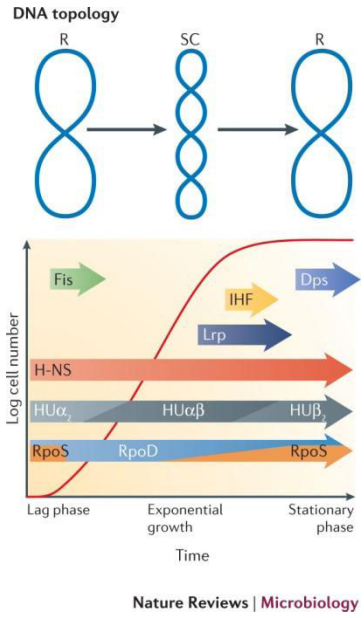


Figure 3 Figure from (Dorman, 2013) showing the variation of the level of supercoiling and of NAPs composition as a function of growth phase. Negative supercoiling increases in exponential phase, when transcription is maximal, and then decreases in stationary phase. These transitions are accompanied by changes in protein abundance: FIS is present only in exponential phase, while for instance Dps appears only in late stationary phase. In exponential phase the main sigma factor is σ^{70}, encoded by RpoD, while at entry into stationary phase the amount of σ^s increases.

In summary, the *Escherichia coli* chromosome is a dynamically organized structure whose state depends on the activity of several proteins, on global cellular processes (such as transcription or DNA replication) and on the general physiological state of the cell (ATP level). This dynamic and highly

flexible organization allows the cell to rapidly adapt and survive to environmental changes, as I will describe in the next chapter.

2 Adaptation

E. coli is able to exploit the information included in the chromosome in order adapt to different growth conditions. Below I will describe the changes in macromolecular composition, gene expression and partitioning of resources that characterize the response of the cell to a changing environment.

2.1 Adaptation to different growth rates

It has been known since the 1920s that bacteria change their size in response to growth conditions, i.e. becoming bigger in exponential growth and smaller when the growth slows down (HENRICI, A. T., 1928). In 1952 Wade observed different phases in the growth of *E. coli* after dilution in a fresh medium, and reported the existence of a lag phase, a phase of rapid cell division, a phase of constant division rate and growth rate (exponential phase) and a phase when the growth rate decreases (Wade, 1952). In agreement with the results obtained by Henrici, he reported an increase in cell weight during lag phase followed by a decrease when the cells decrease their growth rate. An increase in DNA and RNA amount per cell was observed in lag phase, when cells are adapting to fresh medium, followed by a decrease when cells enter exponential growth (Morse and Carter, 1949). Schaechter et el. studied the size, the DNA and RNA content and the number of origin per cell for *S. typhimurium* in balanced growth in different growth media (Schaechter et al., 1958). They showed that at a given temperature, the average mass as well as the amount of DNA and RNA and the number of origin per cell depend exponentially (with different exponents) on the growth rate, irrespectively on the kind of nutrients supplied. Cooper and Helmstetter in 1968 derived an equation connecting DNA amount in exponential phase and growth rate (Cooper and Helmstetter, 1968). The number of copies of a gene g in the cell is defined by the equation

$$g = 2^{\wedge\ [C*(1-m')+D]/\tau}$$

with τ the doubling time, m' the distance of the gene from the origin of replication (0 in the origin, 1 in the terminus), C the time necessary for the completion of DNA replication, and D the interval between the end of DNA replication and cell division. They were able to explain how bacteria can duplicate

faster than the time required for DNA replication and cell division by introducing the idea of overlapping rounds of DNA replication, i.e. a replication process is started before the previous one is completed (see Figure 4).

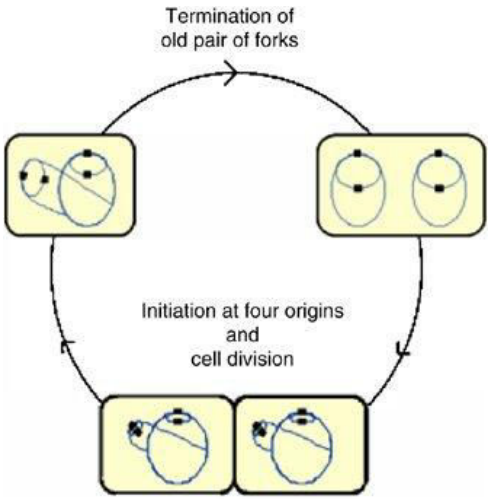


Figure 4 Figure from (Fossum et al., 2007) describing the overlapping rounds of DNA replication in cells at fast growth. Before the 'old' DNA replication round is completed, another one begins such that the two daughter cells inherit a chromosome where replication is already ongoing. In this way, the cell can divide faster than the time required for DNA replication.

Doubling time (min)	τ=100	τ=60	τ=40	τ=30	τ=24	
Protein/cell	5,6	8,7	13	18,9	25	10^8 aa residues
RNA/cell	3,7	7,3	14,3	24,4	39	10^7 nucl residues
DNA/cell	1,6	1,8	2,3	3	3,8	Genome equiv.
Mass/cell	0,85	1,49	2,5	3,7	5	OD_{460}/ 10^9 cells
Protein/mass	6,5	5,8	5,2	5,1	5	10^{17} aa/ OD_{460}
RNA/mass	4,3	4,9	5,7	6,6	7,8	10^{16} nucl/ OD_{460}
DNA/mass	18,3	12,4	9,3	8	7,6	10^8 genomes/ OD_{460}
RNAP/cell	1,5	2,8	5	8	11,4	10^3 RNAP/cell
%RNAP transcribing stable RNA	24	36	56	68	79	
[ppGpp]	55	38	22	15	10	pmol/ OD_{460}

Table 1 Cellular parameters varying as a function of growth rate, from (HANS BREMER, 1996). At increasing growth rate there is an increase in the amount of total protein, RNA and DNA per cell. The cellular mass increases as well at fast growth, such that the concentration of the protein is almost constant at different growth rates. The concentration of the alarmone ppGpp decreases at increasing growth rates.

Bremer reviewed the growth rate dependence of cellular parameters (HANS BREMER, 1996). The most important of them are summarized in Table 1. It should be noted the strong increase in cell mass

at faster growth rates and the increasing fraction of RNAPs transcribing stable RNA at fast growth results in an increase in stable RNA concentration. The nucleotide ppGpp regulates gene expression during the stringent response (Gentry and Cashel, 1996). The importance of ppGpp in the regulation of stable RNA synthesis was shown by Hernandez and Bremer (Hernandez and Bremer, 1993). In their work, they measured the ratio between stable RNA synthesis rate and the total RNA synthesis rate at different growth rates for a wild type strain and for a ppGpp-deficient strain. They found that in the wild type strain this ratio was increasing with increasing growth rate, while in the mutant this ratio remained constant. Therefore they concluded that the regulation of stable RNA synthesis requires ppGpp. The effects of ppGpp on ribosomal and gene expression will be discussed in more detail in paragraph 2.4.

2.2 Adaptation after a shift from one growth medium to another

Several cellular parameters are affected upon a shift to a richer or a poorer growth medium. For instance, it was shown that after a shift to a richer growth medium there was an increase in FIS level (Ball et al., 1992), in the number of ribosomes (Dennis and Bremer, 1974), in the number of RNAP molecules (Iwakura and Ishihama, 1975) and in the level of negative supercoiling (Balke and Gralla, 1987). Kjeldgaard et al. shifted cultures of *S. typhimurium* from poor to rich growth medium (upshift) and from rich to poor growth medium (downshift) (Kjeldgaard et al., 1958). They discovered that as a response to the upshift, RNA synthesis is immediately increased, mass synthesis changes quickly and in 5 minutes reaches the typical level for fast growth, while it takes 20 minutes for DNA synthesis to shift to the new rate, and 70 minutes for cell division to adapt to the new rate. The same lag in tuning cell division rate to the new growth conditions was obtained for *E. coli* by Ball et al. (Ball et al., 1992). Cooper suggested that the reason for this lag was to be found in the constant C an D period (Cooper, 1969). New replications initiated after the shift will result in cell division only after the C+D period (~70min). Brunschede et al. studied the effect of an upshift on macromolecular composition in *E. coli* (Brunschede et al., 1977). Their results show that stable RNA, DNA and protein amounts increase such that the ratios ΔRNA/ΔProtein and ΔDNA/ΔProtein are constant.

After a down-shift, synthesis of mass and RNA stops for 30-40 min while cell division and DNA synthesis continue until the cell mass is reduced to an half (Kjeldgaard et al., 1958). The rate of synthesis of RNA then increases up to the value characteristic of the slower growth rate, while the DNA and protein synthesis reacts more slowly to the downshift (Neidhardt and Magasanik, 1960). The synthesis of RNAP subunits behaves like the RNA synthesis rate, dropping rapidly and then regaining the rate typical of the new growth medium (Iwakura and Ishihama, 1975). The importance of the changes in these cellular parameters is underlined by the recent observation that upon a change in growth phase gene expression is mainly dependent on the global state of the cell (free RNAP, free ribosomes, nutrient availability and gene copy number) than on the activity of specific transcription factors (Berthoumieux et al., 2013).

2.3 Adaptation to changes in growth phase: entry into stationary phase

As it was described above, *E. coli* is able to adapt for rapid growth in presence of increased nutrients. In this section I will describe how *E. coli* adapt in order to survive to starvation. When E*. coli* faces an environment with a lack of nutrients, it activates a starvation-induced reaction that decreases the metabolism and increase the resistance to external stresses (Siegele and Kolter, 1992). Upon starvation, cells enter stationary phase and become smaller and spherical via the induction of the morphology regulator *bolA* (Lange and Hengge-Aronis, 1991). The synthesis of peptidoglycan during amino acid starvation is subjected to stringent control by ppGpp and continues at 30% of the rate of growing cells (Ishiguro and Ramey, 1976). The cell wall has a different structure from the cell wall synthesized during exponential (or rapid) growth and is less sensitive to degradation and to autolysis induced by penicillin (Tuomanen et al., 1988). When ppGpp accumulation is prevented by chloramphenicol treatment (Sokawa and Sokawa, 1978), there is no increased resistance to autolysis and the starved cell lyses when peptidoglycan synthesis is inhibited (Kusser and Ishiguro, 1985). The bacterial surface also changes upon starvation (Kjelleberg and Hermansson, 1984; Nyström et al., 1988). Upon entry into stationary phase, glycogen is synthesized and accumulated (Romeo and Preiss, 1989). Under conditions of starvation, when no net synthesis of protein is possible, the cell is

nevertheless able to synthesize new proteins for adaptation by degrading already existing proteins (Reeve et al., 1984a) and RNA (Mandelstam and Halvorson, 1960). This synthesis of new proteins is necessary for cell survival (Reeve et al., 1984b), since a starved cell has no means to quickly respond to the environment, and therefore has to be prepared in advance to possible hostile conditions. For this reason, about 30 proteins are upregulated and synthesized during the first 3-4 hours of stationary phase (Groat and Matin, 1986), and some of them are typical of other stress responses, such that ethanol exposure, heat-shock and SOS response (Gottesman, 1984). Coherently with this observation, starved cells display higher resistance to heat shock, oxidative stress, and osmotic challenge than exponentially growing cells (Jenkins et al., 1988, 1990, 1991). The induction of expression of these proteins depends both on changes in the level of negative supercoiling and on changes in the RNAP composition.

Coherently with a slowdown of metabolism, ATP concentration decreases while the cell enters into stationary phase, decreasing slowly to about 40% and then dropping faster (Chapman et al., 1971), with this steep decrease in ATP concentration corresponding to a loss of viability of the cells. In concomitance with the decrease in ATP levels, also the negative superhelicity decreases, because gyrase needs ATP (Balke and Gralla, 1987). This decrease of negative supercoiling is fundamental for the recognition of promoter sequences by the σ^s subunit of RNAP (Kusano et al., 1996), since the promoters recognized by σ^s and σ^{70} have an almost identical consensus (Gaal et al., 2001) and several promoters can be recognized by both holoenzymes (Tanaka et al., 1993). One example is the *osmE* gene, a gene induced by σ^{70} upon osmotic shock during exponential phase and by σ^s at the entry into stationary phase (Conter et al., 1997). Cells lacking the sigma factor σ^s maintain a rod shape in stationary phase (Lange and Hengge-Aronis, 1991) and are not resistant to thermic or osmotic shocks (McCann et al., 1991). The amount of sigma factor σ^s increases in the cell facing stresses or entering into stationary phase (Loewen et al., 1993), and it was shown that σ^{70} and σ^s compete for a limited amount of RNAP in stationary phase (Farewell et al., 1998). Factors determining this competition are again the level of supercoiling, which triggers the shift between the σ^{70} and the σ^s usage (Bordes et al., 2003), and ppGpp which reduces the ability of σ^{70} for core binding (Jishage et al., 2002). In addition to the role played in the competition between sigma factors, ppGpp is required for σ^s synthesis (Gentry et al., 1993; Lange et al., 1995). In the next paragraph I will discuss more in detail the importance of ppGpp during stringent response.

2.4 Adaptation to amino acids starvation: the stringent response

When *E. coli* faces the lack of an essential amino acid, the cell reacts with a severe restriction of ribosomal synthesis, named stringent control. RNA accumulation is strongly reduced (Sands and Roberts, 1952), the rate of RNA synthesis rapidly decreases to 30% of the level in exponential growth and RNA is enriched in mRNA with respect to ribosomal RNA (Lazzarini and Dahlberg, 1971). Upon amino acids starvation, there is a sudden accumulation of ppGpp (Cashel, 1969; Erlich et al., 1971).

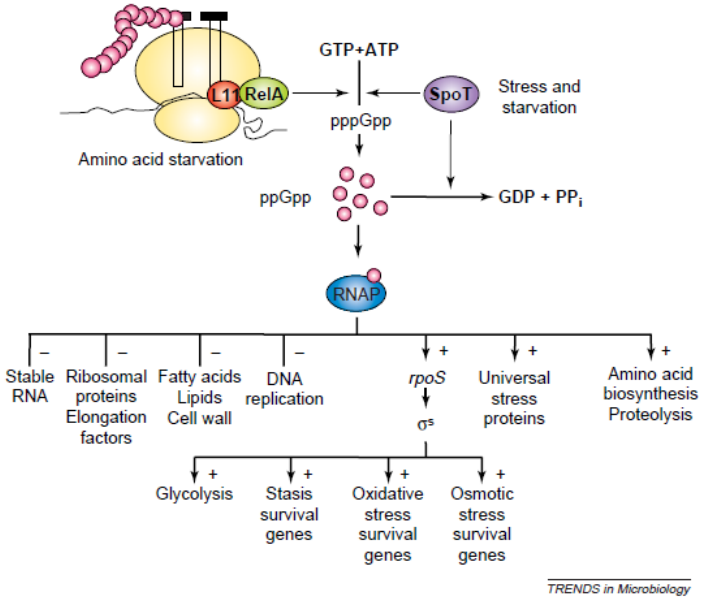


Figure 5 Figure from (Magnusson et al., 2005) describing the regulation by ppGpp upon amino acid starvation. ppGpp is produced by RelA and SpoT and binds to RNAP molecule resulting in particular in a repression of stable RNA synthesis and in the induction of *rpoS*, which encodes for sigma factor associated with stress response in *E. coli.*

This nucleotide, produced via RelA and SpoT (Xiao et al., 1991), binds to the β and β' subunits of core RNA polymerase (Chatterji et al., 1998; Toulokhonov et al., 2001). This results in an inhibition of rRNA synthesis (Gourse et al., 1986; Travers, 1976; Zhou and Jin, 1998), due to a decrease in the stability of the complex formed between a ribosomal promoter and the σ^{70} holoenzyme (Gourse et al., 1998). Barker et al. showed that ppGpp decreases the stability of open complexes, therefore inhibiting transcription from ribosomal promoters that already make short-lived open complexes due to the presence of the GC-rich discriminator region (Barker et al., 2001a, 2001b). The rate of stable RNA synthesis decreases from 1.0 at a ppGpp concentration of 0, to 0.24 at saturating concentrations of

ppGpp (Ryals et al., 1982). The increase in ppGpp therefore causes the dissociation of RNAP from ribosomal promoters, resulting in an increased availability of polymerase for metabolic promoters (Zhou and Jin, 1998). In addition to this passive regulation of metabolic promoters, ppGpp and DksA work together to increase transcription from amino acids promoters in response to nutrient limitation (Paul et al., 2004, 2005).

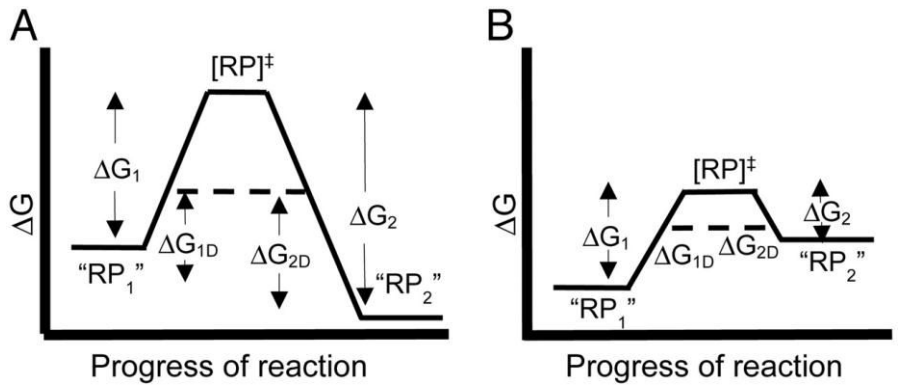


Figure 6 Figure from (Paul et al., 2005) showing the effect of ppGpp and DksA on the stability of RNAP-promoter complex for metabolic (A) and ribosomal promoters (B). The decrease of the barrier between the two intermediate states I1 and I2 in the isomerization process is beneficial for the metabolic promoters, because the step to the I2 state is facilitated and I2 state is still stable, being its energy lower than the one of I1. For ribosomal promoters the decrease in the Energy barrier between intermediate 1 and 2 has a negative effect, because it destabilizes the state I2, therefore resulting in a decrease of ribosomal promoter expression.

The destabilization of the RNAP-promoter complex was described by Paul et al., for two intermediates separated by a transition state RP‡ that the promoter and the polymerase have to form on the pathway to open complex formation (Paul et al., 2005). For a metabolic promoter, there is a high barrier in the free energy to overcome the transition state and get to the second complex, but since the free energy of this second complex is lower than the one of the first complex, the second complex is stable. The effect of ppGpp and DksA is to lower the barrier between the two complexes (i.e. the free energy of the transition state). In case of a metabolic promoter, this facilitates the step to the second complex and the reaction is still stable. In this way, ppGpp and DksA increase the transcription from metabolic promoters. In contrast, for a ribosomal promoter the energy of the transition state is also lowered, making the step to the second complex easier, but the free energy of the second complex is higher than the free energy of the first one, therefore the second complex is unstable. This instability is

worsened when ppGpp and DksA decrease the free energy of the transition state, inhibiting transcription and releasing RNAP molecules from ribosomal promoters as a result.

In addition to the release of polymerase from ribosomal promoter (with the consequent passive up-regulation of metabolic promoters) and the direct activation by ppGpp and DksA, the effect of ppGpp on ribosomal and metabolic promoters has been explained in a different way via the Michaelis–Menten model described below (Figure 7 and Box 2).

A

$$E + S \underset{k_r}{\overset{k_f}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P$$

B

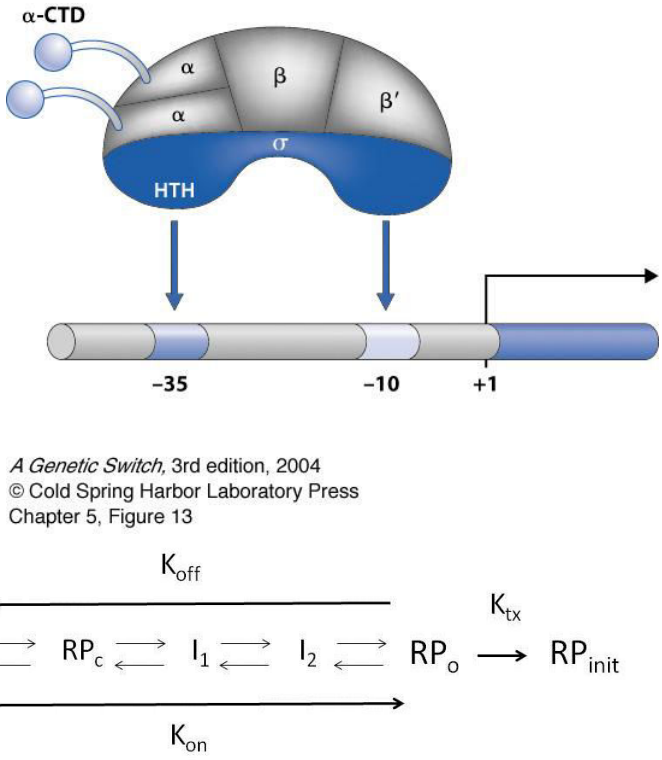


Figure 7 A) Enzymatic reaction process: an enzyme E binds to the substrate S at rate k_f, dissociates at rate kr and once bound to the substrate converts the substrate in product P at a rate k_{cat}. B) The complex of reactions from the recognition of the promoter by the RNAP to the open complex formation and the initiation of transcription can be modeled in the same way via the Michaelis-menten equation.

Box 2 Isomerization

RNAP recognize a promoter sequence via and interaction of the sigma subunit with the -10 and -35 regions to form a closed complex RPc. At this stage, either the polymerase falls from the sequence or there is a series of conformational changes (isomerizations) both in RNAP and in the DNA, leading through different intermediate states (I_1, I_2) first to the opening of the DNA double helix and then to the formation of a stable open complex (RPo) (Saecker et al., 2011). From this point, the transcription process begins.

In 1913 Michaelis and Menten derived a mathematical model to describe the process of the enzymatic reaction in which sucrose is hydrolyzed into fructose and glucose (Michaelis and Menten, 1913). The process can be described as in Figure 7, with E the concentration of the enzyme, S the concentration of the substrate, P the concentration of the product and k_{cat}, k_f and k_r the kinetic constants of the process. At equilibrium, the velocity of the reaction is defined by

$$v0 = \frac{Vmax\ [S]}{(Km + [S])}$$

with

$$Km = \frac{kr + kcat}{kf}$$

V_{max} the maximal reaction velocity occurring when the entire amount of enzyme is bound to the substrate. K_m is the concentration of the substrate for which the velocity of the reaction is half of the maximal velocity V_{max}, and is the inverse of the affinity of the substrate for the enzyme. The ratio V_{max}/K_m can be considered as the catalytic ability of the enzyme, i.e. the efficiency of the transformation of the substrate. To be noticed that different values of K_m and V_{max} can result in the same catalytic ability.

The process of RNAP binding to the promoter in order to form an open complex and to start transcription can be regarded as a Michaelis-Menten process, where the parameters K_m and V_{max} depend on the promoter characteristics (Figure 7). A high value of K_m means that the affinity of the promoter for RNAP is low, therefore promoters approach saturation only at high concentration of RNAP. On the contrary, promoters with a low K_m are saturated at a lower RNAP concentration and their expression at fast growth (when the amount of RNAP is high) is limited by V_{max}. The parameter V_{max} in this case represents the frequency of transcription events in the case of promoter saturated by RNAP.

It was shown that the activity of ribosomal promoters increases at increasing growth rate, while it decreases for other promoters (Liang et al., 1999a; Miura et al., 1981). Knowing that the amount of free RNA polymerase increases at fast growth rate (HANS BREMER, 1996), this result means that

ribosomal promoters are not saturated by RNAP, therefore their activity can increase in presence of a higher amount of RNAP, while the other promoters are saturated at a lower level of RNAP, thus they cannot increase indefinitely their activity at increasing growth rate. Following this idea, Jensen and Pedersen proposed that the promoter strength V_{max}/K_m was low for stable RNA promoters, with both V_{max} and K_m high, while the promoter strength was high for metabolic promoters, with low values of V_{max} and K_m (Jensen and Pedersen, 1990). Therefore a ribosomal promoter has low affinity for RNAP (unstable open complex) and a high rate of transcription, while open complex at metabolic promoters is stable and the frequency of initiation of transcription is low. In this way Jensen and Pedersen proposed an explanation of the different effect of ppGpp on ribosomal and metabolic promoters. Since the increase in ppGpp enhances pauses of RNAP during transcription (Kingston et al., 1981), the amount of free RNAP decreases in presence of ppGpp. For this reason, in presence of ppGpp the expression of metabolic promoters that are saturated at low concentration of RNAP is favored with respect to the one from ribosomal promoters that need a higher concentration of free RNAP to approach their maximal rate.

Klumpp and Hwa analyzed the different explanations for ppGpp effect on metabolic and ribosomal promoters (Klumpp and Hwa, 2008). In their model, RNAP sequestration due to pauses in the transcription does not have an effect strong enough to inhibit the expression of ribosomal promoters. A post-transcriptional auto-regulation of ribosomal proteins binding to their own mRNA and suppressing translation in case of lack of rRNA is a more efficient mechanism to ensure inhibition of ribosomal protein expression (Fallon et al., 1979; Scott et al., 2014). Similarly, the increase in free polymerase due to ppGpp's effect on the unstable open complex at the ribosomal promoters can only partially explain the induction of metabolic promoters in presence of ppGpp. Therefore they propose that the leading force in the upregulation of metabolic promoter is the direct induction operated by ppGpp and DksA together.

Box 3 Effects of Chloramphenicol on ribosome regulation

As described previously, ppGpp binds to RNA polymerase, causing a decrease in rRNA synthesis. In case of a reduced amount of rRNA, ribosomal proteins instead of binding to rRNA and form ribosomes bind to their own mRNA, repressing r-protein synthesis (Dean and Nomura, 1980; Yates et al., 1980). In this way the equilibrium stable RNA-ribosomal proteins is maintained. When chloramphenicol is added in sub-lethal concentration to an exponentially growing *E. coli* culture, the growth rate decreases (Harvey and Koch, 1980) because chloramphenicol binds to ribosomes inhibiting translation (Wolfe and Hahn, 1965). In this situation, the internal concentration of amino acids increases rapidly due to translation inhibition (Kurland and Maaløe, 1962) with a consequent rapid decrease in the level of ppGpp (Gallant et al., 1972) and derepression of ribosomal promoters. For this reason, when cells are exposed to chloramphenicol there is an accumulation of stable RNA and ribosomes (Shen and Bremer, 1977a, 1977b).

In summary, the cell is able to regulate gene expression in response to the environmental conditions. This regulation is achieved both via digital control, i.e. specific transcription factors, and via global regulators, such as supercoiling level, NAPs or sigma factors, defining the analog control of cellular functions. Both these digital and the analog types of information are enclosed in the DNA molecule, its sequence and its structure, thus creating a self-referential and connected system allowing the cell to respond to perturbations as a unit (Muskhelishvili et al., 2010).

3 Economy in a cell

As it was shown in the previous section, the cell needs to adapt its macromolecular composition to the growth rate and to divide its resources among different processes. One of the parameters changing with the growth rate and affecting gene expression is the amount of RNA polymerase_(HANS BREMER, 1996). In 2008 Klumpp and Hwa proposed a model to calculate the growth-rate dependence of free RNA (Klumpp and Hwa, 2008). They divided the total number of RNAPs into different classes: the number of RNAPs transcribing mRNA (Nm), RNAPs transcribing rRNA (Nr), RNAPs that are bound to DNA nonspecifically (Ns), immature RNAP (Ninterm) and RNAP that are free to begin transcription (Nfree). Using different values from previous works (HANS BREMER, 1996), they calculated the growth rate dependence of these different classes (see table 2).

Growth rate (db/hr)	**0,6**	**1**	**1,5**	**2**	**2,5**
Ntotal	1500	2800	5000	8000	11400
Nm	184	341	457	599	709
Nr	59	194	568	1302	2598
Nfree	97	238	476	718	868

Table 2 Growth rate dependence of the number of total RNAP, of RNAP transcribing mRNA, of RNAP transcribing ribosomal RNA and of RNAP free in the cytoplasm, as derived from (Klumpp and Hwa, 2008). At fast growth, there is a strong increase in the number of RNAP molecules transcribing rRNA, while the number of molecules transcribing mRNA increases less.

It should be noted the strong increase at increasing growth rates in the number of RNA polymerase molecules transcribing ribosomes, while the number of RNA polymerases transcribing mRNA has just a 4-fold increase. This difference gives an idea of how the cell divides its resources between ribosomes and protein synthesis at different growth rates. For ribosomal promoters, there is an increase in promoter activity at fast growth (Zhang and Bremer, 1995) due to activation by FIS (Zhang and Bremer, 1996), to the release of the repression by ppGpp (Zhang et al., 2002) and to increased negative supercoiling level (Travers and Muskhelishvili, 2005a). This partitioning of polymerase has important consequences on gene expression. Liang et al. measured beta-galactosidase expression

driven by three different constitutive promoters and showed that at slow growth there was an increase in beta-galactosidase activity with respect to fast growth (Liang et al., 2000). Wanner and co-workers had already shown that at rapid growth the expression of several promoters was decreased, with the exception of the strong promoter *lacUV5* that maintains the level of expression constant as a function of growth rate (Wanner et al., 1977). Klumpp et al. proposed a simple model to explain this dependence of expression on the growth rate (Klumpp et al., 2009). They use the value for the amount of free RNAP calculated before (Klumpp and Hwa, 2008) in order to compute the transcription rate per copy of a gene, and then they defined the protein concentration p for a constitutive (i.e. non-regulated) gene by

$$p = \frac{g * \alpha m * \alpha p}{\beta m * \beta p * V}$$

with V the cell volume, g the gene copy number, αm the transcription rate, αp the translation rate per mRNA, βm the degradation rate of mRNA and βp the one for protein. Since βm and αp are considered independent on the growth rate, the dependence on the growth rate lies in g, αm, βp and V. On the considered range of growth rate, g increases approximately 2.5 fold and αm less than 3-fold, while the volume and the degradation rate of the protein (approximated as the dilution rate for a stable protein) increases both 4- fold from slow to fast growth. Therefore a decrease in protein concentration is unavoidable (see Figure 8A). An almost constant level of protein concentration as a function of growth rate in this model is possible only for a repressed promoter.

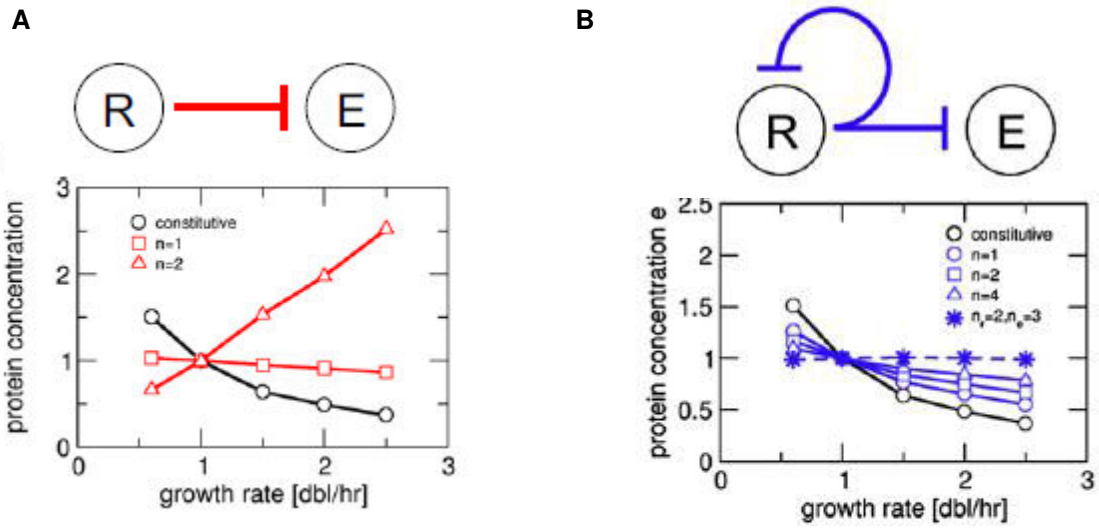


Figure 8 Plots from (Klumpp et al., 2009) showing the growth rate dependence of protein concentration for a constitutive promoter (black) and a promoter repressed by a constitutive promoter (red) for different degrees of cooperativity in the repression, and protein concentration for a promoter repressed by a negatively autoregulated promoter (blue). When cooperativity is required for repression, the expression of the target gene increases at fast growth because the repressor protein is diluted (A). For the expression of a target repressed by an autorepressed gene, this derepression at fast growth is less prominent because the concentration of the regulator tends to be constant at the different growth rates.

After having addressed the question of the growth rate dependence of metabolic genes expression, Scott et al. studied the connection between growth rate and repartition of resources for ribosomal and metabolic genes (Scott et al., 2010). It has already been shown by Ryals et al. that at fast growth almost all the RNA in bacteria is stable rRNA and tRNA, while at slow growth or in presence of high levels of ppGpp almost the 75% of RNA is mRNA (Ryals et al., 1982). Following this result, Scott et al. found a linear relationship connecting growth rate and the fraction of RNA over total protein via the translational capacity of the organism k_t

$$r = r_0 + \lambda/k_t$$

and via the nutrient quality of the medium at changing translational inhibition

$$r = r_{max} - \lambda/k_n$$

RNA fraction therefore increases both at increasing growth rate and at increasing level of translational inhibition, while metabolic proteins display an opposite behavior (sees Figure 9)

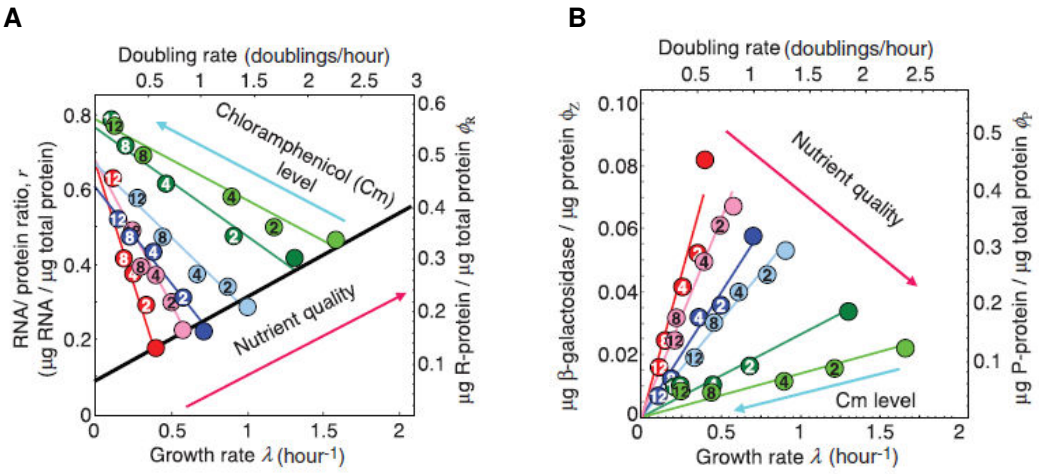


Figure 9 Figures from (Scott et al., 2010) describing the growth rate dependence of the fraction of RNA over total protein (A), and of the fraction of protein expressed by a constitutive promoter over total protein (B). At increasing nutrient quality, the fraction of RNA increases while the concentration of the constitutively expressed protein decreases (pink arrow). At fixed growth medium, an inhibition of translation via chloramphenicol (blue arrow) results in an increase of the fraction of RNA over total protein and a decrease of the concentration of the protein synthesized from the constitutive promoter.

They explained the increase in RNA/protein by a compensation for the reduced translational capacity, and the decrease in protein synthesis by the consequent increased number of RNAPs synthetizing ribosomes. This competition between ribosomal and metabolic proteins is due to the limited amount of resources in the cell (see Figure 10) that have to be divided between housekeeping genes (Q), ribosomal and translational proteins (R) and metabolic proteins (P). An increase in the fraction of resources devoted to ribosomal genes happens at the expense of the metabolic proteins fraction. The repartition of resources necessary to maximize the growth rate in a given environment is the result of the balance of the amino acid influx, guaranteed by the transport proteins belonging to the P fraction, and the number of ribosomes consuming amino acids in order to produce proteins (Scott et al., 2014). If the amino acids level is low, the protein translation rate decreases while if the amino acids pool increases, there is an increase in the ribosome fraction R and a consequent reduction of transport proteins (and of nutrient influx) that guarantees the flux balance of the system.

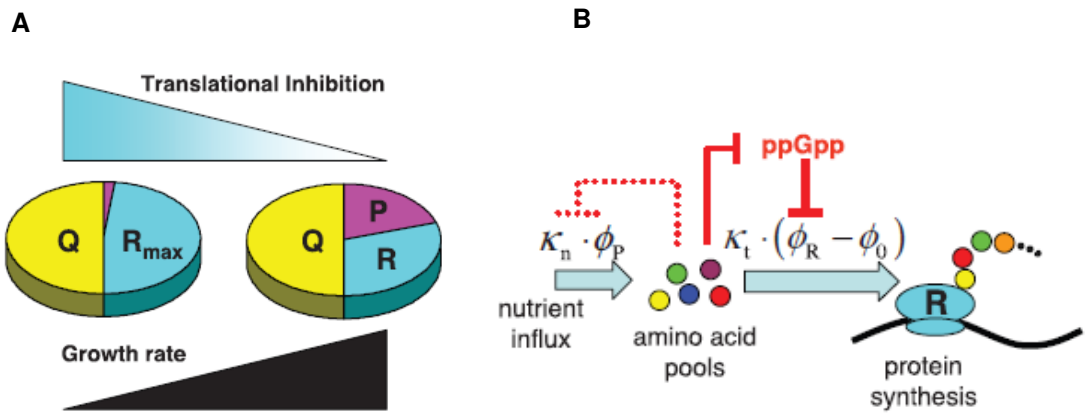
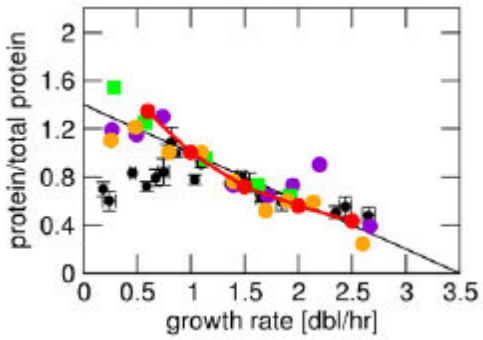


Figure 10 A) Schematic representation from (Scott et al., 2010) of the repartition of transcription and translation resources between housekeeping genes Q, ribosomes and translational proteins R and metabolic proteins P. At increasing translational inhibition, the growth rate decreases and the fraction allocated for the R part increases. B) The level of amino acids in the cell determines both the rate of the nutrient influx and the amount of ppGpp, which regulates ribosomes synthesis.

Box 4 A strange constitutive: *lacUV5*



(Klumpp et al., 2009)

In this figure the black dots show the expression of the *lacL1* promoter from (Wanner et al., 1977), a constitutive *lac* promoter mutant whose activity does not decrease as a function of growth rate like the other promoters plotted (Klumpp et al., 2009). Wanner et al. studied the expression of the wild type *lac* promoter and of several mutants under different growth conditions (Wanner et al., 1977). In particular, they studied the expression of *lacL1* and *lacUV5* mutants, which are not activated by CRP-cAMP. For the *lacL1* promoter, they found that for growth rates faster than one doubling per hour, the β-galactosidase synthesis decreases at increasing growth rate. On the contrary, the β-galactosidase synthesis driven by the UV5 promoter was not dependent on the growth rate. UV5 has the same -35 region and the same sub-optimal spacer of the wild type promoter (Stefano and Gralla, 1982), but in addition it has mutations resulting in a consensus -10 region (Malan and McClure, 1984). Due to this fact, UV5 is a strong promoter with a high rate of isomerization (de Crombrugghe et al., 1984) and frequent initiation (McClure, 1980). At strong promoters, as soon as a polymerase elongates beyond the promoter, another polymerase is bound and starts another RNA chain (McClure, 1980). This mechanism was observed for rRNA operons (Pace, 1973). Wanner et al. suggested that the strength of the UV5 promoter was the reason of the observed a constant β-galactosidase concentration as a function of growth rate, because strong promoters compete well against ribosomal promoters for free polymerases and therefore are able to take advantage of the increased amount of polymerase at fast growth. This different dependence of protein synthesis as a function of growth rate for constitutive promoters with different promoter strength is not taken into account in the previously mentioned model by Klumpp et al. (Klumpp et al., 2009).

In summary, in this chapter I present the models that have been proposed in order to give a quantitative description of resources partitioning (RNAP, ribosomes) that could explain the observed dependence of gene expression on growth rate. The role of promoter strength in the growth rate

dependence of gene expression is a player that has not been considered in the model, and that I will study further (see results 6.2.3).

4 Uncovering the Influence of chromosomal position on gene expression

In eukaryotes, the organization of DNA in tightly condensed (heterochromatin) or lightly condensed (euchromatin) structures has a clear influence on gene expression, since the degree of DNA compaction in the gene neighborhood determines the accessibility to the transcriptional machinery (Li and Reinberg, 2011; Voss and Hager, 2014; Zlatanova et al., 2000). It is less clear whether this influence of chromosomal position on gene expression also exists in bacteria. A clear effect on gene expression of the distance from the origin of replication has been widely shown in literature, but other facts such as the uneven distribution of protein binding sites, AT-content as well as the tendency of co-regulated genes to lie one in proximity to the other suggest that the position in the chromosome can play a more subtle role than mere gene abundance. First, I will describe the effect of DNA replication on gene dosage, and the consequence on gene expression. Then I will describe the results pointing to the possibility of an influence of chromosomal position on gene expression beyond gene abundance.

4.1 Gene dosage effect

DNA replication in *E. coli* begins at one origin (von Meyenburg et al., 1978) and proceeds bidirectionally towards the terminus (Prescott and Kuempel, 1972). As already mentioned, it was shown by Cooper and Helmstetter that in fast growing bacteria there are multiple overlapping replication rounds (Cooper and Helmstetter, 1968). This implies that the chromosome in cells at fast growth rate is highly branched, leading to a higher amount of genes located near the origin in comparison to genes that are close to terminus, while at slow growth this difference in gene abundance is lower (Bipatnath et al., 1998). This gene copy number effect has been regarded as the only effect of chromosomal position in gene expression. Back to the ‘70es, Chandler and Pritchard evidenced a proportionality between gene copy number and amount of enzyme produced in steady state (Chandler and Pritchard, 1975). In *Salmonella typhimurium*, the orientation of the traslocated gene did not affect gene expression, and the level of expression from all the insertions followed the trend dictated by gene dosage (Schmid and Roth, 1987). Sousa et al. measured the expression for the

LacZ gene driven either by the P*sal* promoter (involved in the biosynthesis of salicyclate) or by the *gyrB* promoter at different chromosomal positions by using mini transposons insertion (Sousa et al., 1997). They aimed to detect a difference in *gyrB* expression due to differences in the local levels of supercoiling in the chromosome, but all the positions appeared to be affected in the same way by changes in the supercoiling, and the only effect that was shown was again the decrease in gene expression due to a decrease in gene dosage while approaching to the terminus. Block et al. built a synthetic network made up of the LacI repressor on one side, and on the other the YFP reporter gene under the control of PLlacO-1 promoter (Lutz and Bujard, 1997) with translation regulated by the T7 10 5' UTR sequence (Block et al., 2012). They showed that changes in chromosomal position or in gene orientation or in the distance of the target from the repressor did not affect gene expression, and that the only variation in gene expression as a function of chromosomal position was due to changes in gene abundance and in the local effect when the reporter was not isolated from the environment. Ying et al. inserted the fluorescent reporter GFP gene in different position in the chromosome either under the control of a native promoter located in the chosen position, or replacing the native promoter with the P*tet* promoter (Ying et al., 2014). They studied the level of expression during exponential phase in presence or in absence of the repressor TetR. Their results for the P*tet* promoter show a clear scaling in gene expression with the increasing distance from the origin, compatible with a decrease in gene copy number, both with and without the repressor. For the native promoters they didn't find a decrease in gene expression with increasing distance from the origin, therefore they concluded that not all the highly expressed promoters are located near the origin.

4.2 The *Escherichia coli* chromosome is not uniform

4.2.1 The intrinsic structural properties of DNA sequences

Differences in the chromosomal structure could result in differences in gene expression according to the gene's position. Satchwell et al. studied eukaryotic sequences and found certain triplets that correlate with the DNA wrapping around the histone (Satchwell et al., 1986). They defined the 'position preference' as the measure of the frequency in which the triplet is specifically positioned on the nucleosomal DNA with respect to the surface of the core protein. The concept of 'position preference'

was extended by Willenbrock and Ussery in order to describe the general property of DNA to be wrapped around proteins (Willenbrock and Ussery, 2007). They measured position preference in several bacterial genomes (*E. coli*, *P. aeruginosa*, *G. sulfurreducens*, *C. jejuni*, *B. subtilis*, *S. cerevisiae*) and discovered that highly expressed genes such as ribosomal RNAs and ribosomal proteins are located in regions with low values of position preference, i.e. in less flexible regions of the chromosome. They postulated that these regions were more accessible to RNAP and other elements of the transcriptional machinery. Pedersen et al. studied DNA curvature, DNA flexibility, and DNA stability of several prokaryotic genomes, discovering that the terminus was characterized by high level of curvature, low flexibility, and low DNA stability (Pedersen et al., 2000). They also showed the existence of several regions with strong curvature, probably acting as domain boundaries, and they observed, in agreement with Willenbrock and Ussery, that the regions in which ribosomal genes are located display similar structural parameters, pointing to the importance of chromosomal structure in gene expression. Sobetzko et al. measured the melting temperature distribution in the chromosome of γ-proteobacteria and found a gradient from high average negative DNA melting in the origin to low average negative melting energy in the terminus (Sobetzko et al., 2013). In addition, they were able to show that depending on the growth phase, the cell is using genes with different melting temperature: at the beginning of exponential phase the cell activates genes that are close to the origin and that have lower negative melting temperature, while in later stage of growth the activated genes have a higher negative melting temperature and are located farther from the origin of replication. This pattern of expression reflects the decreasing level of energy and supercoiling in the cell going from exponential to stationary phase.

4.2.2 Dynamics *in vivo*: diffusion of DNA and of proteins, heterogeneity in the cytoplasm, gradients in local concentrations

The motility of tagged DNA as a function of macrodomain position was studied by Javer et al., and it was observed that on short time scales (ms-s) the diffusion of loci located in different chromosomal positions was dependent on the chromosomal coordinate (Javer et al., 2013). Protein diffusion is also influenced by the location of the encoding gene. Kuhlman and Cox used single-molecule measurement of the protein expressed by LacI fused with a fluorescent protein gene in different chromosomal position to test the influence of the encoding gene position on protein diffusion (Kuhlman

and Cox, 2012). They grew cells in different growth media, fixed them, imaged in the microscope and found that when the encoding gene was located on a plasmid, the protein was evenly distributed, while for chromosomal insertions the protein location was correlated with the gene position. Moreover, they were able to show that the distribution of the protein was highly influenced by the degree of chromosome compaction: in stationary phase, when DNA is highly dense, the protein is excluded from the volume occupied by DNA and it is located towards the cell poles, while it can diffuse more freely during exponential phase. Wunderlich and Mirny proposed that a transcription factor searches its target by either sliding on DNA, or with a free diffusion in the cytoplasm (Wunderlich and Mirny, 2008). If the protein is unevenly distributed, and if it is not very abundant, a rapid regulation of the target requires proximity between regulator and target (Kolesov et al., 2007). With an *in silico* analysis of the regulatory network, Hershberg et al. demonstrated that genes regulated by the same transcription factor tend to be adjacent to each other, and that a transcription factor generally regulates targets close to its encoding gene (Hershberg et al., 2005) or located at regular distances, possibly due to solenoidal organization of the chromosome (Képès, 2004).

4.2.3 Patterns in the genome suggest possible three dimensional organizations of the nucleoid

Periodicity in the localization on the chromosome was found also for most essential and highly transcribed genes (Mathelier and Carbone, 2010; Wright et al., 2007), and co-regulated operons were found to be transcribed in divergent direction, in such a way that their regulatory upstream sequences overlap (Warren and ten Wolde, 2004). Horizontally transferred genes tend to cluster along the genome close to macrodomain barriers (Zarei et al., 2013), and genes regulated by H-NS as well as genes co-regulated by H-NS and StpA are found in clusters located mainly in the terminus and at macrodomain barriers (Srinivasan et al., 2013). Therefore, it seems that one of the forces driving gene location on the chromosome is the co-regulation and the proximity to regulators. Montero Llopis et al. demonstrated that mRNA remains localized near the encoding gene, therefore suggesting that for interacting proteins the proximity of the encoding genes could be an advantage in order to facilitate their interaction and assembly (Montero Llopis et al., 2010).

4.2.4 Important genes are arranged in the chromosome according to their temporal pattern of expression

Transcription seems to be the driving force also for spatial arrangement of the genes in the cell. A strain expressing a fusion lac repressor-GFP was engineered in order to localize a plasmid carrying an array of lac operator sites and to compare its position to the one of the same plasmid with a constitutive promoter inserted (Sánchez-Romero et al., 2010). It was shown that while the first plasmid was evenly distributed in the cytoplasm, for the second one carrying the constitutive promoter an aggregation of plasmids was located toward the cell poles, suggesting an advantage for gene expression deriving from a specific localization in the cell. Sobetzko et al. gave a global picture of both the proximity target-regulator, confirming the results described in the previous paragraph, and the precise location of important genes in the chromosome (Sobetzko et al., 2012). They analyzed the chromosomal position of important genes in γ-Proteobacteria, and found that their distance from the origin of replication (see Figure 11) reflects the temporal pattern of their expression during cell growth: genes required in exponential phase (such as *fis*, *dnaA* and the ribosomal genes) are located toward the origin, while genes connected with stationary phase or stress response (such as *topA*, *topB* or *fnr*) are closer to the terminus. They also found that *rpoD*, coding for σ^{70}, is closer to the origin than *rpoS*, gene encoding for the stationary phase-related σ^{s}. In addition, targets for σ^{70} are located mainly close to the origin, while the targets for σ^{s} are found near to the terminus. The origin is also enriched in genes upregulated by gyrase (Jeong et al., 2004).

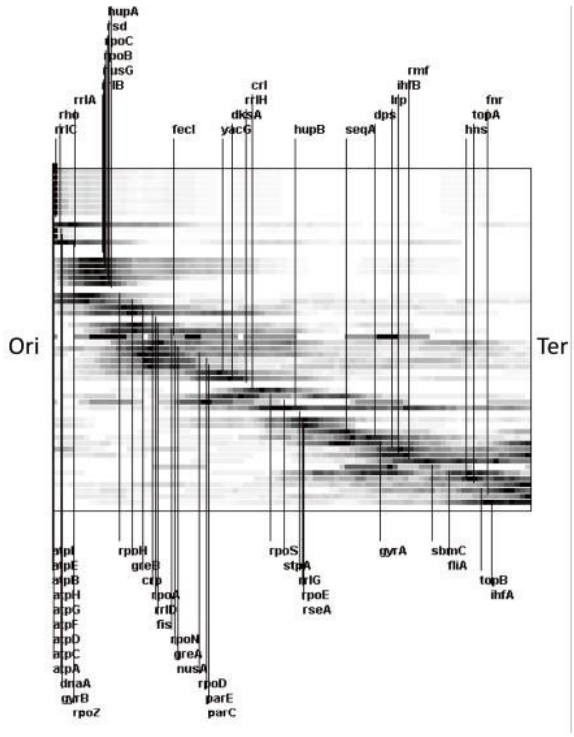


Figure 11 Figure from (Sobetzko et al., 2012) representing the conservation of the distance from the origin in γ-Proteobacteria of important genes such as ribosomal genes, NAPs, genes coding for sigma factors.

4.2.5 The role of dynamic assemblies in the change of nucleoid organization as a function of growth rate: the transcription foci.

At fast growth, different chromosomal positions are characterized by the formation of transcription foci (Cabrera and Jin, 2003). This precise co-localization of polymerase vanishes at slow growth, under stringent response or when transcription is inhibited by rifampicin, showing that the distribution of the polymerase changes with the transcription activity in the cell. A similar result was obtained by measuring RNAP localization on the chromosome by chromatin immunoprecipitation and microarrays (Grainger et al., 2005). It was shown that at fast growth the polymerase is mainly bound to ~90 regions in the chromosome, corresponding to genes involved in translation and in ATP synthesis. Upon inhibition of transcription by the addition of rifampicin, or upon addition of IPTG and salicylic acid, the RNAP localization was reorganized accordingly (for example, upon addition of IPTG the polymerase was found bound to *lacZ*). Therefore, the distribution of RNAP polymerase is dynamic, and the proximity to a highly expressed gene could be an advantage due to a higher amount of polymerase molecules nearby.

4.2.6 Heterogeneous distribution of NAPs

The distribution of NAPs binding sites along the chromosome is also uneven. In the terminus, there is an increase in the total length of H-NS binding regions when the cell proceeds from exponential to stationary phase (Zarei et al., 2013). Grainger et al. showed that half of the binding sites for FIS, H-NS and IHF were found in non-coding regions of *E. coli* genome, probably responding to an architectural function, while there is an overlap of the binding sites for FIS and H-NS in coding regions, pointing to a combined regulation of gene expression (Grainger et al., 2006). The NAP HU was found to bind non specifically to the chromosome, with a particular preference toward A/T-rich regions (Prieto et al., 2012). More in general, protein occupancy is not homogeneous along the chromosome: instead, the existence of extended regions of protein binding was shown (Vora et al., 2009). These regions could be distinguished in highly transcribed (heEPODs) and transcriptionally silent ones (tsEPODs), with the latter being characterized by high AT-content and by the binding of H-NS, FIS and IHF. Therefore a specific position of the gene in the chromosome could be characterized by a different protein binding, resulting in a specific gene expression.

Even with this clear heterogeneity of the chromosome, the influence of chromosomal position on gene expression in *E. coli* has been elusive. One step in the direction of discovering this influence has been recently made by Busby's group (Bryant et al., 2014). They inserted *lac* promoter driving the expression of a fluorescent reporter gene in different position in the chromosome, and measured the fluorescence per unit of OD for cultures in exponential phase in minimal media supplemented with IPTG. Their results show a strong impact of chromosomal position on the reporter gene expression, with fluorescence concentration varying over a ~300-fold range. These differences were not due to gene dosage effects, but they were instead to be ascribed to different protein occupancy levels, to gyrase effects and to transcribing neighboring genes. This interesting result gives new stimulus for further research, in order to study to which extent chromosomal position affects gene expression for different promoters and in different growth conditions and growth phases.

5 Experimental approach:

We decided to study the effect of chromosomal position on the expression of fluorescent reporter gene under the control of HNS promoter, of the ribosomal rrnBP1 promoter and of the phage promoter P5 in different growth conditions and at different growth phase of the cell.

5.1 Phns

H-NS is an abundant nucleoid associated protein that acts as a global repressor (Hommais et al., 2001) especially in silencing horizontally acquired genes (Oshima et al., 2006; Zarei et al., 2013). It binds preferentially to AT-rich sequences (Dame et al., 2001) and to high affinity sites (Bouffartigues et al., 2007) and can form heteromeric (Williams et al., 1996) or oligomeric structures (Dorman et al., 1999). These structures contribute in shaping the chromosome (Dame et al., 2000; Noom et al., 2007) and in bridging distal regions creating foci (Wang et al., 2011). H-NS protein is expressed at a relatively constant level in *Escherichia coli* (Free and Dorman, 1995). Overproduction of H-NS is lethal for the cell (Spurio et al., 1992) due to excessive compaction of the chromosome, and the lack of H-NS has a strong impact on several aspects of cell life (Giangrossi et al., 2005; Soutourina et al., 1999). For this reason, H-NS expression needs to be regulated: it was shown that FIS binds to different sites on H-NS promoter (Falconi et al., 1996) and contrasts the repression that H-NS exerts on its own promoter (Falconi et al., 1993). As a response to cold shock, CspA enhances H-NS expression (La Teana et al., 1991). H-NS expression is repressed by its paralogue StpA (Williams et al., 1996). The two proteins show almost the 70% of sequence similarity (Sonnenfield et al., 2001; Zhang et al., 1996), StpA expression can complement Δ*hns* phenotype (Shi and Bennett, 1994) and the double mutation Δ*stpA*Δ*hns* strongly affects cell growth (Sonden and Uhlin, 1996).

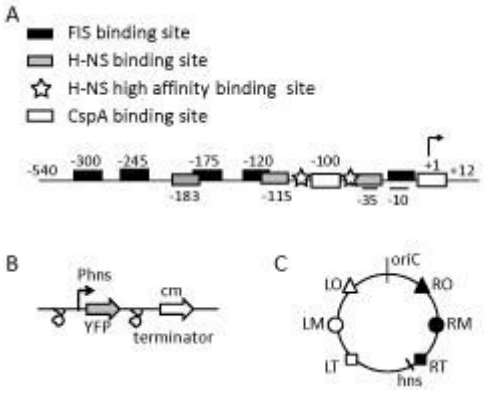


Figure 12 A) Schematic representation of the *hns* regulatory region. One of the binding sites of H-NS overlaps with the -35 region, therefore repressing its own expression, while the binding of FIS to the promoter region prevents H-NS repression. B and C) The Phns+YFP reporter construct studied in this work and the six chromosomal positions in which it has been inserted.

5.2 *rrnB*P1

In *Escherichia coli* there are seven ribosomal genes, located near the origin of replication (Figure 13). The activities of the different promoters are similar, after correction for gene abundance effects (Condon et al., 1992) and they are tightly regulated (Schneider et al., 2003).

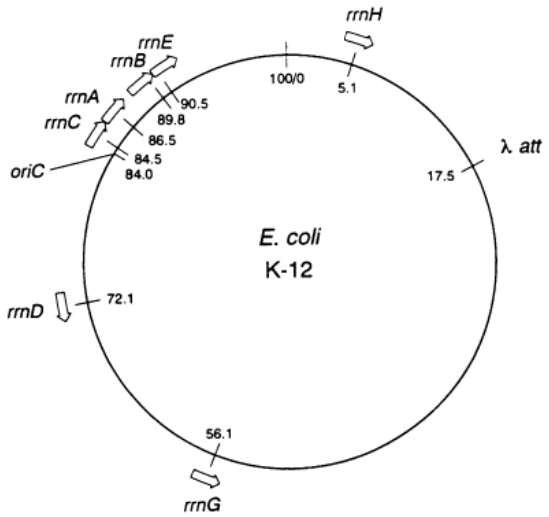
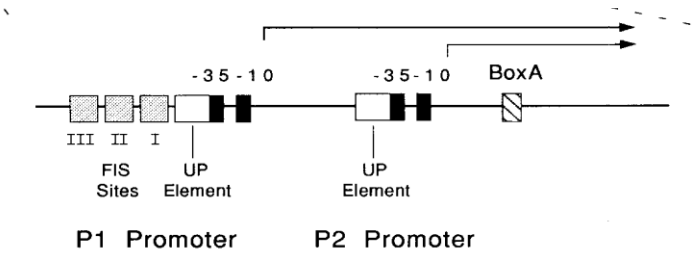


Figure 13 Figure from (Condon et al., 1992) showing the seven ribosomal genes located mainly near the origin of replication. This proximity to the origin of replication guarantees high levels of expression at faster growth due to the increase in gene copy number.

There are two promoters responsible for transcription of rRNA operon, named P1 and P2 (Gilbert et al., 1979) (Figure 14A). P2 maintains a basal level of expression and is not affected by growth rate

(Dennis et al., 2004), while P1 expression increases with increasing growth rate (Sarmientos and Cashel, 1983) and is sensitive to ppGpp (Liang et al., 1999a). Gourse et al. showed that the growth rate dependence of P1 promoter was due to a specific sequence located inside the promoter itself (Gourse et al., 1986), and this sequence was identified in the GC-rich discriminator located between the -10 element and the transcription start point (Travers et al., 1986). The discriminator is responsible for regulation by ppGpp (Lamond and Travers, 1985) and for supercoiling sensitivity (Travers and Muskhelishvili, 2005a). A sequence located upstream the -35 element, named UP element, was found to be necessary for optimal promoter activity (Gourse et al., 1986; Lamond and Travers, 1983). The sequence named box A, situated downstream the promoter P2, is required for efficient rRNA chain elongation (Gourse et al., 1986). FIS binds upstream the UP element and increases P1 activity ~40 times, and P2 activity ~5 times, at increasing growth rate (Zhang and Bremer, 1996), while H-NS represses ribosomal promoter expression (Afflerbach et al., 1998; Schröder and Wagner, 2000). In our analysis we focused on the short version of rrnBP1 promoter, without the FIS binding sites (Figure 14B), that is less active but nevertheless growth-rate dependent and stringent control dependent (Bartlett and Gourse, 1994; Josaitis et al., 1995).

A



B

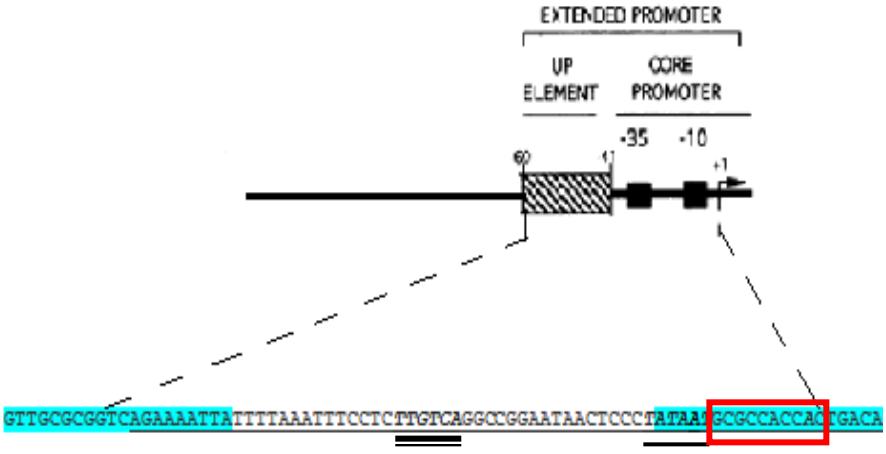


Figure 14 Figure adapted from (Bartlett and Gourse, 1994). A) The structure of the P1 and P2 promoters driving ribosomal genes expression. Both P1 and P2 have the UP element, required for recruiting RNAP, and P1 has three FIS binding sites upstream the UP element. B) Close-up on the P1 promoter and on the sequence without the FIS binding sites we used in our analysis. The UP element, the -10 and -35 regions as well as the GC-rich discriminator region are indicated.

5.3 T5-P5

As a constitutive promoter, we decided to study the strong virulent T5 coliphage promoter P5 (Wang et al., 2005). This promoter has both a consensus -10 and -35, as well as an optimum 17bp spacer, and it was demonstrate to have a high affinity for *E. coli* RNAP (Gentz and Bujard, 1985). In the P5 sequence below the -10 and -35 regions are shown in bold.

5'-ACAACATCTAAGAGAAAAATTATA**TTGACA**TCTGCCCTTGAATAAGC**TATAAT**AGTAGTCTT
AGTTAGAGAAGGAGGGTATAAT-3'

We expect the P5 promoter to have similar characteristics with the lacUV5 and ribosomal P2 promoters described above (Wanner et al., 1977).

6 Results

In this chapter I will describe the results obtained by studying *Phns* expression as a function of chromosomal position, which we conveyed in a paper, under submission, reproduced below, and then I will present the results obtained with the P1 and P5 reporter insertions. The work on the *Phns* reporters began in the laboratory of Prof. Georgi Muskhelishvili at Jacobs University, where the strains were engineered by Michael Berger (PostDoc), Veneta Gerganova and Viktoras Lisicovas (Master students) while the work on the P1 and P5 promoters was carried out at the LBPA where I engineered the strains, with the help of Damel Mektepbayeva (Master student).

The most striking result for the *Phns* study was the identification of a chromosomal position characterized by a stronger repression by H-NS than the others. By carefully considering the data from exponential to stationary phase and the data in exponential phase for different growth rates, we were able to define three different levels of sensitivity to H-NS repression according to chromosomal position. We therefore conclude that the position in the chromosome for a gene regulated by the global regulator H-NS can confer to the target a different degree of repression conditional on the growth phase and environmental parameters affecting H-NS activity. On the other hand, gene expression for P1 and P5 scales with the gene copy number as a function of chromosomal position at different growth rates and in different growth phases. Different kinds of experiments have allowed us to put in evidence the different behaviors of the two promoters upon nutritional upshift or upon inhibition of translation, but only by removing the neighboring kanamycin gene of the reporter cassette were we able to identify a positional effect on gene expression. Our results therefore show a dependence of gene expression as a function of chromosomal position that is different for the different promoters.

6.1 Gene regulation by H-NS as a function of growth conditions depends on chromosomal position in *E. coli*.

6.1.1 Abstract

Cellular adaptation to changing environmental conditions requires the coordinated regulation of expression of large sets of genes by global regulatory factors. It remains to be established whether in bacterial cells there is an influence of chromosomal position on the efficiency of these global regulators. Here we show for the first time that genome position can affect transcription activity of a promoter regulated by the H-NS protein, a global regulator of bacterial transcription and genome organization. We have used as a local reporter of H-NS activity the level of expression of a fluorescent reporter protein under control of an H-NS regulated promoter (Phns) at different sites along the genome. Our results show that the activity of the Phns promoter depends on whether it is placed within the AT-rich regions of the genome that are known to be bound preferentially by H-NS. This modulation of gene expression moreover depends on the growth phase and the growth rate of the cells, reflecting the changes taking place in the relative abundance of different nucleoid proteins and the inherent heterogeneous organization of the nucleoid. Genomic position can thus play a significant role in the adaptation of the cells to environmental changes, providing a fitness advantage that can explain the selection of a gene's position during evolution.

6.1.2 Introduction

The effect of genomic position on the regulation of gene expression is a long standing question that has been addressed in both eukaryotic and prokaryotic cells. While gene expression in eukaryotes is clearly affected by the position due to the level of compaction of chromatin (Voss and Hager, 2014), for bacterial cells this influence of chromosomal position on gene expression has been more elusive. Since the pioneering work of Chandler and Pritchard in 1975 (Chandler and Pritchard, 1975) it was clear that due to the presence of overlapping DNA replication rounds, there are more copies and therefore more expression for a gene close to the origin of replication than for one close to the terminus (Cooper and Helmstetter, 1968). This has been proposed to be the main reason for the conservation of gene position (Couturier and Rocha, 2006; Sobetzko et al., 2012), but other factors could be involved.

Cellular adaptation to changing environmental conditions requires the coordinated regulation of expression of large sets of genes. This regulation can take place via the activity of specific transcription factors and/or through the effects of global regulators. The latter include small metabolites, such as cAMP, ppGpp or c-di-GMP, specific sigma factors, the set of abundant nucleoid proteins (NAPs) and changes in DNA topology (Blot et al., 2006; Bradley et al., 2007; Geertz et al., 2011). Recent high throughput studies have identified the genes whose expression is affected by these different regulatory factors and the binding sites of nucleoid proteins along the genome (Cho et al., 2008; Grainger et al., 2006; Kahramanoglou et al., 2011; Lucchini et al., 2006; Oshima et al., 2006; Peter et al., 2004; Wade et al., 2007), as well as the presence of clusters of co-regulated genes along the genome (Scolari et al., 2011; Vora et al., 2009; Zarei et al., 2013). These findings suggest that the level of expression of a given gene may also depend on its local environment and thus its position in the genome, an idea that is also supported by the high level of conservation of a gene's position with respect to the distance from the origin of replication in the family of gammaproteobacteria (Sobetzko et al., 2012).

In order to determine the extent to which chromosomal position can influence the regulation of expression of a given gene, one can place the same reporter construct at different sites along the genome. Previous studies using this approach have reported that the basal activity as well as the level of induction or repression of a reporter gene by a specific transcription factor were not affected by the position (Block et al., 2012; Ying et al., 2014). This is in agreement with earlier reports using other promoters in E.coli and other bacterial species (Dryselius et al., 2008; Schmid and Roth, 1987; Sousa et al., 1997; Thompson and Gasson, 2001). In order to address whether the regulation of gene expression by a global regulator such as H-NS was also independent on genomic position we have inserted a construct consisting of an H-NS dependent promoter (Phns) regulating YFP expression at six different mirror sites across the two replichores. The use of this promoter not only allows us to probe the local level of H-NS activity, but also allows us to obtain some information on how the hns gene itself might be regulated and the effect it would have if moved away from its evolutionary conserved position in the genome, near the terminus of replication.

H-NS is a well characterized, highly abundant (~20000 copies), nucleoid organizing protein that can affect the expression of hundreds of genes (Dorman, 2007). Notably, gene regulation by H-NS plays an important role in the response to stress, such as acid or cold shock (Giangrossi et al., 2005; La

Teana et al., 1991). H-NS has a high affinity for AT-rich regions (Tanaka et al., 1991) and can also recognize a specific consensus sequence (Lang et al., 2007). The high affinity sites can act as nucleation sites for further oligomerisation. This oligomerized state can repress transcription either by trapping RNAP already bound on the DNA or inhibiting RNA polymerase (RNAP) binding to a promoter sequence (Dame et al., 2006; Hommais et al., 2001; Maurer et al., 2009; Schröder and Wagner, 2000), thus H-NS has been shown to also play a role in preventing transcription of spurious RNA from -10 sequences found within AT-rich regions (Singh et al., 2014). Moreover, the ability of the H-NS protein to bridge different DNA regions together contributes to compaction of the nucleoid (Dillon and Dorman, 2010).

6.1.3 Materials and Methods

Chromosomal insertions

The hns promoter (+ 12 to -540 bp from the start site of transcription) was cloned upstream the YFP gene and the gene coding for resistance to chloramphenicol. Upstream of the promoter and downstream of the YFP gene T1 terminators from the E. coli rrnB coding sequence were added in order to stop transcription from RNA polymerases coming from neighboring genes. The construct was amplified by PCR and inserted in the E. coli CSH50 strain in six different chromosomal positions (Table 1) following the protocol by Datsenko and Wanner (Datsenko and Wanner, 2000). The insertions were made between two convergent genes, in order to avoid perturbations due to promoter regions of neighboring genes. More details about these constructions can be found in (Berger et al., 2014).

Plate reader Assay

Cultures were grown overnight in LB supplemented with chloramphenicol (20µg/ml), at 37°C in a shaking incubator. Using the automated pipetting workstation Biomek 3000 (Beckman Coulter), samples were diluted 1:10000 into a 96well plate, and grown in triplicates at 37°C or 30°C inside the plate reader Victor3 (Perkin Elmer), with shaking. Samples were covered with mineral oil (Sigma

Aldrich) in order to avoid evaporation. OD600 measurements and fluorescence measurements (excitation filter = F485/14, emission filter=F535/40) were taken for each well every 5 minutes. The growth media used were M9 minimal media supplemented with glucose 0.4%, casaamino acids 0.5%, casaamino acids 0.2%, glucose 0.4% and casaamino acids 0.5%.

The data obtained from the plate reader measurements was analyzed using a custom Matlab (MathWorks) program. The value of the OD600 for each well was normalized for the value of the well containing only the growth medium. The fluorescence measurements were normalized by subtracting the fluorescence of the wild type strain that does not contain the fluorescent reporter gene. Fluorescent protein concentration was calculated as YFP/OD600, and the growth rate α as

$\alpha = \frac{d(OD600)/dt}{OD600}$.

The doubling time is then calculated as $\tau = ln(2)/\alpha$.

Flow cytometer

Cultures were grown overnight in LB supplemented with chloramphenicol (20µg/ml), at 37°C in a shaking incubator, and diluted in the morning 1:250 in M9 minimal media supplemented with the desired nutrients. The strains were grown in flasks at 37°C, shaking. At mid-exponential phase (OD600~0.2) 2 ml samples were harvested, washed with filtered PBS and fixed with 4% formaldehyde (Sigma) at room temperature for 15 minutes, washed again with PBS and then analyzed with a flow cytometer (BD FACS CaliburTM , BD biosciences) using the software BD CellQuest™ Pro.

The voltage for FSC and SSC were chosen so that the bacterial population was entirely on scale on an FSC vs SSC plot. A non-fluorescent bacterial sample was used in order to appropriately set the FL1 voltage. Individual FSC, SSC and FL1 histograms were checked to insure that the bell-shaped populations are not cut off on the display. An event rate of ~1000 events per second was maintained in order to minimize the chance of coincidence and to improve population resolution. In the FSC vs

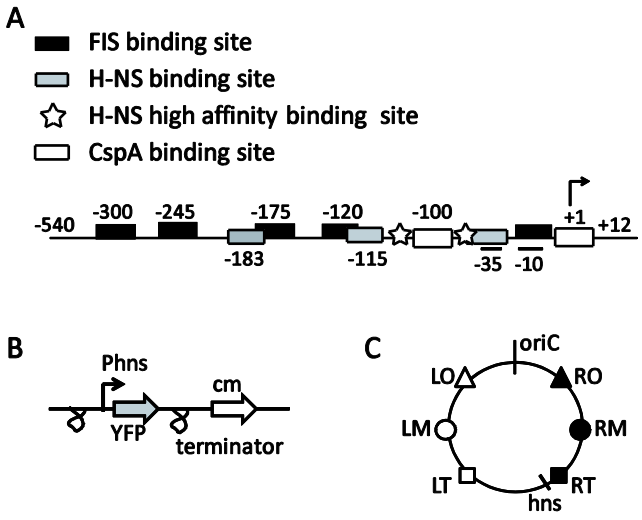
SSC plot a live gate R1 was set around the bacterial population and a total of ~20000 events inside the gate were measured.

6.1.4 Results:

The reporter construction, comprising the Phns promoter upstream of the YFP gene next to an antibiotic resistance cassette, was inserted at six sites along the genome in three sets of mirror sites on each side of the origin of replication (H-NS Fig. 1 and H-NS Table 1). Gene expression from the hns promoter is mainly regulated by FIS and the H-NS protein itself(Falconi et al., 1993, 1996; Ueguchi et al., 1993), therefore these reporter strains can be used to measure the relative changes in activity of these two nucleoid proteins along the chromosome as a function of the growth phase and growth rate. The strains containing the reporter construct in different positions were grown in a 96-well plate overnight in order to monitor the changes in OD and fluorescence as a function of time in growth media of different composition resulting in different growth rates. In order to control for the emergence of heterogeneity in the bacterial population, possibly leading to a decreased average amount of measured fluorescence from YFP, the amount of fluorescence per cell was also measured in parallel experiments by flow cytometry, for cells growing in exponential phase, in a flask, in a shaking incubator, confirming the results obtained in the plate reader (SI Fig. 1).

Insertion	Orientation of P*hns*-YFP	Gene1	Insertion position	Gene2
LO: Left Origin	+1	yhcN	3,383,900	yhcO
RO: Right Origin	-1	ytfL	4,437,900	ytfK
LM: Left Medium	-1	yfiF	2,715,500	Ung
RM: Right Medium	+1	gsK	500,700	ybaL
LT: Left Terminus	-1	yehA	2,185,400	yohN
RT: Right Terminus	+1	yccU	1,027,600	yccV
LT 2	+1	yeeJ	2,050,100	yeeL_1
RT1	+1	ycdT	1,093,500	insEF-4
LT1	+1	yegS	2,167,700	gatR_1

H-NS Table 1 Genomic position of the insertion sites used in this work. Gene 1 and gene2 correspond to the convergent genes upstream and downstream of the reporter construct.

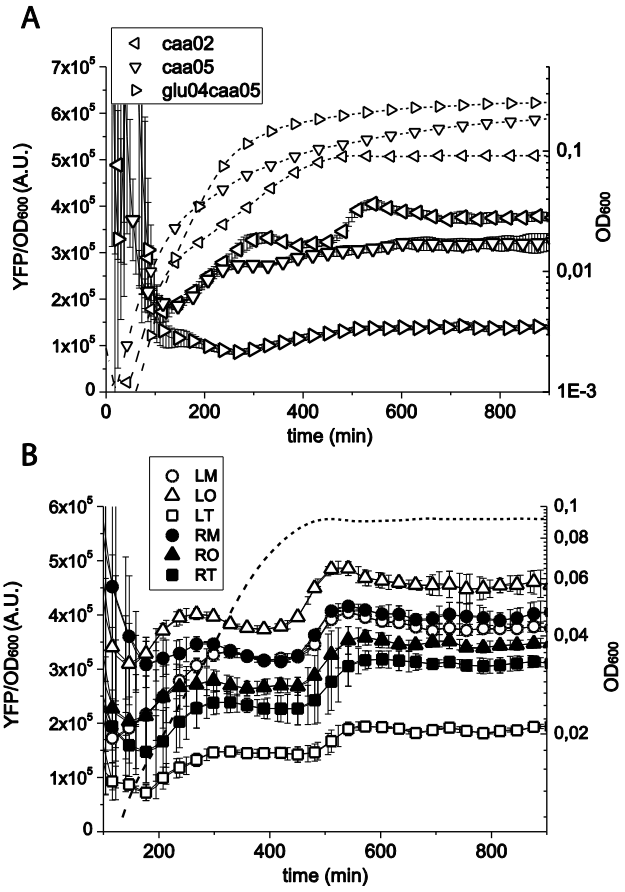


H-NS Figure 1 Schematic representation of the P*hns* promoter and of the insertions in the *E. coli* chromosome. (A) The boxes indicate the binding sites for different proteins in the P*hns* promoter region (striped for FIS, grey for H-NS, white for CspA) as derived from the literature(Falconi et al., 1996; La Teana et al., 1991; Ueguchi et al., 1993). Stars indicate the H-NS high affinity DNA binding sites (Lang et al., 2007). The -10, -35 regions and the transcription starting site, +1, are also annotated. (B) The promoter-*yfp* unit is flanked by two T1 terminators from the *E. coli rrnB* coding sequence. (C) Representation of the six different mirror sites on the *E coli* chromosome in which the *yfp* gene was inserted under the control of the P*hns* promoter next to the gene conferring resistance to chloramphenicol. The symbols used here are the ones used to indicate these positions in H-NS Fig. 2B, H-NS Fig. 5 and H-NS Fig. 8. Details about the insertion positions can be found in H-NS Table 1.

The growth phase dependence of *Phns* promoter activity depends on the growth rate

The results obtained in the plate reader show that the change in YFP concentration as a function of growth phase depends on the growth rate (H-NS Fig. 2A). In the richer medium (glu04caa05) the protein concentration is lower than in the other growth media (caa02 and caa05) and it remains more or less constant in the growth curve. On the other hand, when the bacteria grow in the poorer media there is an increase in protein concentration as the growth rate slows down during the transition to stationary phase. During growth in caa02 there is a second increase in concentration as the cells enter stationary phase. The high temporal resolution of the plate reader measurements allows us to obtain a measure of the change in promoter activity and growth rate as a function of time. These results indicate that during this growth phase transition, the growth rate slows down before the change in promoter activity does, resulting in a net accumulation of YFP (SI Fig. 2). Therefore a specific induction of hns promoter activity upon entry into stationary phase needs not to be invoked in order for this protein accumulation to take place. These results can provide an explanation for the different

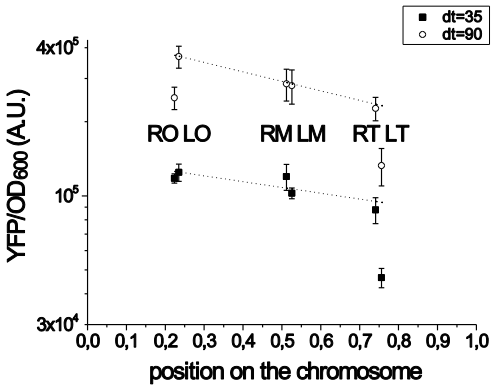
profiles in H-NS expression observed in previous studies (Atlung and Ingmer, 1997; Dersch et al., 1993; Free and Dorman, 1995; Ueguchi et al., 1993).



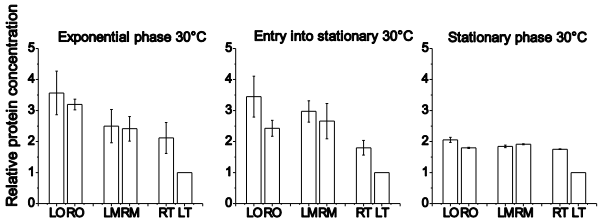
H-NS Figure 2 Change in OD and YFP concentration obtained from the plate reader measurements. The average protein concentration as a function of time is shown from technical triplicates within a single experiment, which is one out of three independent experiments. The dashed lines represent the change in OD, while the continuous lines the change in YFP concentration. (A) The growth phase dependence of YFP concentration depends on the growth rate. At fast growth (glu04caa05) there is no accumulation of the protein while entering in stationary phase, while at slow growth (caa02) the amount of protein per unit of OD increases as cells approach to stationary phase. This difference in promoter expression as a function of the growth condition doesn't depend on the chromosomal position of the insertion. (B) YFP concentration depends on the chromosomal position of the gene. In the same growth conditions (caa05 at 30°C), the amount of protein concentration is higher for insertions near the origin of replication (as expected due to gene copy number). It is possible to see that for the strain with the insertion in the Left Terminus (LT, white squares), the concentration is considerably lower than the one in the Right Terminus strain (RT, black squares), even though for both the positions the gene copy number is the same.

For most of the sites, comparison of YFP concentration as a function of genomic position in exponential phase shows a difference between the sites that can be explained by the differences in gene copy number expected from the DNA replication process (H-NS Fig. 3). Interestingly, there is also a difference in gene expression between the sites that are equidistant from the origin, notably

between Left Terminus (LT) and Right Terminus (RT) and Right Origin (RO) and Left Origin (LO). The latter difference becomes evident especially at slow growth and upon entry into stationary phase (H-NS Fig. 3 and H-NS Fig. 4).



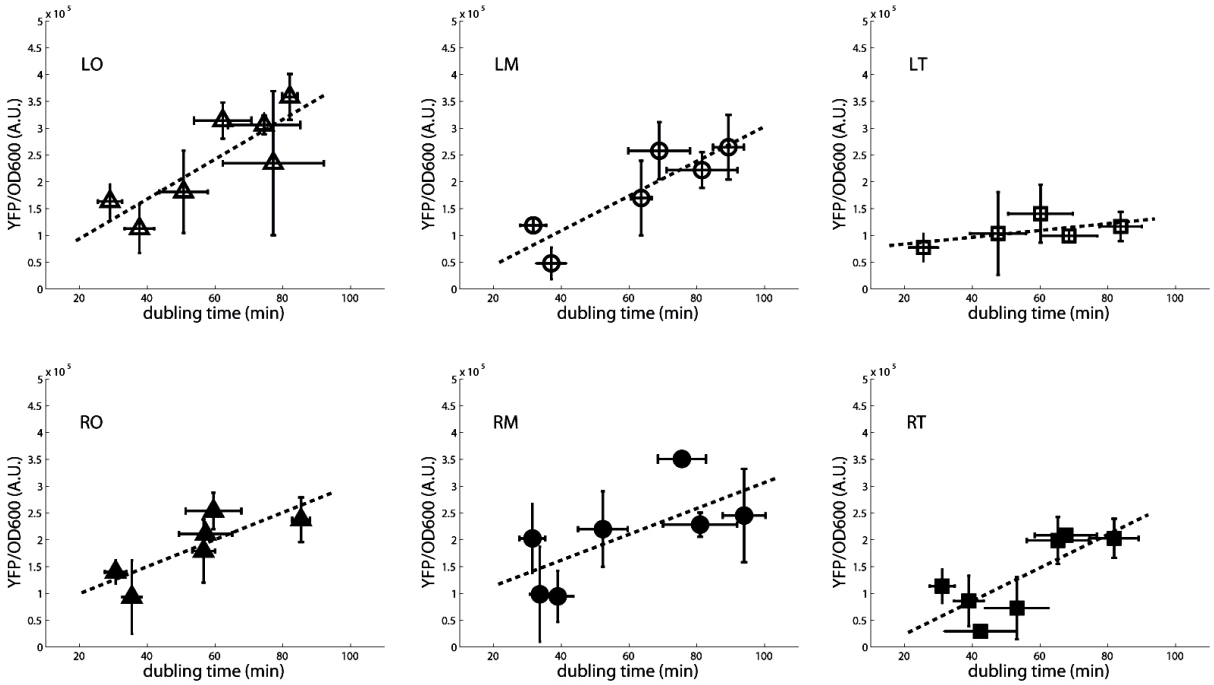
H-NS Figure 3 The difference in YFP concentration in exponential phase at two different growth rates can be explained for most positions by the change in gene copy number. YFP concentration was measured in mid exponential phase for the six chromosomal insertions at two different doubling times as a function of chromosomal position (0 for the origin of replication, 1 for the terminus). Data are the average of three independent plate reader experiments, the error bar is the SEM. The dotted line is the theoretical dependence of protein concentration as expected by the difference in gene copy number (Cooper-Helmstetter relation) for each growth rate (Cooper and Helmstetter, 1968). The protein concentration for the LT strain is lower than what would be expected as a consequence of gene dosage, both at fast and at slow growth rates. At slow growth, the concentration in RO strain also deviates from the theoretical expectation.



H-NS Figure 4 The difference in YFP concentration is larger at lower temperature and in the transition to stationary phase. The protein concentration was normalized by the LT values for strains in exponential, entry into stationary and stationary phase for three independent experiments, the error bar corresponds to the SEM. Data were taken at the time of maximum growth rate, at the time where the growth rate was half of the maximum and at growth rate equal to zero, respectively. The difference between YFP concentration in LO and RO strains arises at the entry into stationary phase. This is not observed when the experiment is carried out at 37°C (see SI Fig. 3). In stationary phase the protein concentration for all the positions decreases. The protein concentration for the LT strain is always lower than in the other strains.

The differences in gene expression at positions equidistant from the origin increase at slower growth rates

By measuring the amount of fluorescence in exponential phase in the different growth media, these experiments show a decrease in YFP concentration in exponential phase as a function of increasing growth rate (H-NS Fig. 5). The faster dilution rate at faster growth results in a lower YFP concentration. This is observed for all six positions, independently of the expected increase in copy number due to DNA replication of the sites near the origin. H-NS Fig. 3 shows in fact that the change in copy number can explain the differences in gene expression between the different positions at the different growth rates. This is consistent with all positions having the same promoter activity that in addition does not change significantly with growth rate. The activation of the Phns promoter by FIS in exponential phase at fast growth (Falconi et al., 1996) doesn't seem sufficient to counteract the dilution rate. There is however one strain that does not follow this trend, the one where the reporter is inserted at the LT position. In this case the concentration of YFP remains almost constant as a function of growth rate.



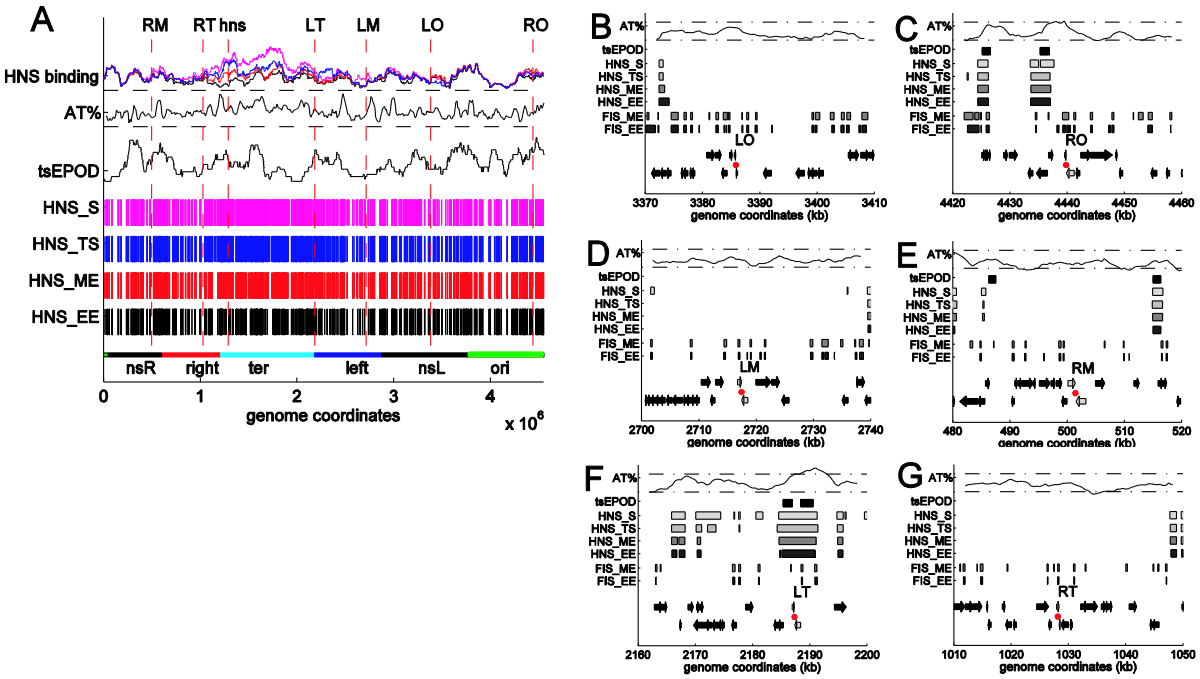
H-NS Figure 5 The change in YFP concentration as a function of growth rate shows an increase in protein dilution at faster growth and a growth rate dependence in the difference between RT and LT. Protein concentration for samples growing in M9 minimal media supplemented with various concentration of glucose and casamino acids at 30°C. The error bars are the SEM resulting from three independent experiments. Data were taken at mid exponential phase. At fast growth there is a low YFP concentration due to a faster dilution rate. For

the LT strain (white squares) the YFP concentration values are low also at slow growth, indicating a stronger repression in this position than at the others.

H-NS binding density due to a higher AT content correlates with promoter repression

The activity of the Phns promoter depends on the activation by the FIS protein in early exponential phase at fast growth (Falconi et al., 1996), CspA for induction upon cold shock (La Teana et al., 1991) and on the binding of the H-NS protein itself resulting in repression (Falconi et al., 1993; Ueguchi et al., 1993). Binding of the H-NS protein along the genome is not uniform and changes as a function of the growth phase (Kahramanoglou et al., 2011; Zarei et al., 2013). When the sites of insertion of the reporter construct are mapped on the H-NS binding patterns one can see that those sites that are less expressed (LT and RO) are found in regions with a higher probability of H-NS binding as measured by formaldehyde crosslinking (H-NS Fig. 6 and SI Fig. 4).

Furthermore, it has been shown that the binding of H-NS to the lower affinity sites leading to oligomerisation and repression is temperature dependent (Bouffartigues et al., 2007). Comparing the results obtained at 30°C and 37°C one can see that the differences between LT and RT and RO and LO are greater as the temperature is decreased, again pointing to an H-NS dependent effect (compare H-NS Fig. 4 and SI Fig. 3). Finally, the loss of the difference between the expression in RT and LT strains in bacteria lacking H-NS confirms that the reduced expression of LT is due to an increased level of repression by H-NS (SI Fig. 5).



H-NS Figure 6 (A) Global view of H-NS binding, AT content and presence of tsEPODs in the *E. coli* chromosome. From the bottom to the top: The macrodomains, as defined by Boccard (Valens et al., 2004). Sites bound by H-NS in early exponential (HNS_EE), mid exponential (HNS_ME), transition to stationary (HNS_TS) and stationary phase (HNS_S) (Kahramanoglou et al., 2011). There is an increase in the number and the length of regions bound by H-NS when approaching stationary phase, especially in the terminus. tsEPODs mapped on the *E. coli* chromosome (Vora et al., 2009). The plot shows the number of genes overlapping with tsEPODs as determined from the NUST software, with multiple sliding windows histogram performed choosing a bin number equal to 32 (Scolari et al., 2012). The AT content is calculated with a sliding window of 50kb with a shift of 10kb. The two horizontal dashed lines correspond to 45 and 55 % AT. The terminus shows a higher AT-content. HNS binding: Sites bound by H-NS in the different growth phases (Kahramanoglou et al., 2011). (B-G) Genomic neighborhood of the chromosomal insertion positions of the reporter construct. From the bottom to the top of each plot: Genes on the lagging and on the leading strand. In grey, the two convergent genes between which the P*hns*-YFP construct was inserted. The site of insertion is shown by a red dot. Position of the sites bound by FIS in early exponential (FIS_EE) and in mid exponential (FIS_ME) phases (Kahramanoglou et al., 2011). There are more sites bound by FIS near the origin (LO and RO, plots on the top) than in the terminus (LT and RT, plots in the bottom). Position of the sites bound by H-NS in early exponential (HNS_EE), mid exponential (HNS_ME), transition to stationary (HNS_TS) and stationary (HNS_S) phases (Kahramanoglou et al., 2011). In the proximity of LT and RO there is an extended region of sites bound by H-NS. Presence of genes identified as transcriptionally silent (Vora et al., 2009). The AT content is calculated with a 4000 bp sliding window with a shift of 500 bases. The two horizontal dashed lines correspond to 45 and 55 % AT. A peak in AT-content is visible near LT and RO.

In order to test whether the difference in expression was specific to the location of the original insertion sites, additional strains were created with insertions at two distances from LT, at about 18000 and 135000 base pairs (LT1 and LT2 respectively) and one additional strain with an insertion at about 66000 base pairs from RT (RT1). The reporters at these sites exhibit a similar level of expression as the original sites (SI Fig. 6), indicating that the differences observed between LT and RT are not limited to the local genomic environment, but that H-NS activity can affect sets of genes within a larger region of the genome. If one takes into account the position of the H-NS binding sites with respect to

the location of the reporter gene (SI Fig. 4) one can see however that RT1 is found in an H-NS rich region compared to RT (H-NS Fig. 6B-G): this results in increased repression upon entry into stationary phase (SI Fig. 6), similarly to what is observed when comparing RO to LO (H-NS Fig. 4).

Previous work had identified regions of the E. coli genome that are rich in DNA-bound proteins and have a lower average level of expression compared to other genes, called tsEPODs, for transcriptionally silent Extensive Protein Occupancy Domains (Vora et al., 2009). A subsequent analysis of the correlation of the genes within these regions with H-NS binding confirmed the colocalization of significant clusters of both tsEPODs and H-NS binding, particularly in regions containing horizontally acquired genes, suggesting that tsEPOD could correspond to H-NS rich regions(Zarei et al., 2013). The map of the sites of insertion of the reporters used here overlapped with both tsEPODs and H-NS binding along the genome. It shows however that the decreased level of expression correlates better with H-NS binding regions than with the presence of tsEPODs. This could be explained in part by the different protocols used to enrich the protein-crosslinked DNA in the tsEPOD (phenol chloroform extraction) and H-NS (antibody pulldown) experiments.

Moving the *hns* gene itself does not have a significant effect on the cell's phenotype or fitness in the short term.

The results obtained with the reporter strains naturally lead to the question of whether the position of the hns gene near the terminus has been conserved through evolution because it results in a specific advantage in growth and thus an increase in fitness. In order to test this we have moved the hns gene at the six genomic positions used here and have tested the ability of these strains to survive the acid and cold shocks that are a hallmark of H-NS activity (Genet et al., 1994; Giangrossi et al., 2005) or their ability to compete with the wild type strain in different growth conditions. In addition, since the Δhns strain is known to lose its ability to swarm (Soutourina et al., 1999), we also carried out swarming assays. All of these experiments showed that the mutant strains grew equally well compared to the wild type (data not shown). This might be due to the fact that, in addition to being a negatively auto-regulated gene, it is also regulated post-transcriptionally by the DsrA RNA resulting in a robust expression level of its product independently from its local genomic environment (Lease and Belfort,

2000). Moreover, H-NS activity is closely coupled with its interaction with other proteins, such as StpA and Hha that can complement its activity (Madrid et al., 2007; Paytubi et al., 2004; Sonnenfield et al., 2001; Ueda et al., 2013; Williams et al., 1996; Zhang et al., 1996). Displacing the hns gene therefore does not have a significant effect, at least in the timescales of a few days probed of our experiments. It is however still possible that there might be an effect on a longer term evolution experiment (Chib and Mahadevan, 2012). We are currently exploring this possibility.

6.1.5 Discussion:

Measuring changes in a transcription regulator activity in vivo: heterogeneous effects along the genome.

In the current work we provide evidence for an uneven effect of H-NS dependent regulation of a reporter gene expression along the genome. Furthermore we have shown that these differences depend on the growth conditions, such as growth phase, growth rate and temperature, reflecting changes in H-NS activity along the genome and thus also changes in nucleoid structure and organization as the cell adapts to different growth environments.

Since the Phns promoter is known to be repressed by H-NS itself via the presence of specific H-NS binding sites and AT-rich sequences upstream of the core promoter region (Falconi et al., 1993; Giangrossi et al., 2001; Lang et al., 2007; Ueguchi et al., 1993), the promoter-reporter construct used here measures the local activity of H-NS. The high affinity of H-NS for AT-rich sequences can result in a higher local concentration of the protein in AT-rich regions of the nucleoid, such as the terminus, and in a higher level of DNA binding cooperativity (Azam and Ishihama, 1999). For example, H-NS is known for playing an important role in silencing horizontally acquired genes, which tend to be more AT-rich than the rest of the genome, and which sometimes also include pathogenicity islands (Dorman, 2013). This level of repression takes place most of the time, except when the genes of the pathogenicity island are induced by specific environmental changes coupled with the activity of a transcription factor (Beloin et al., 2002; Kane and Dorman, 2011; Prosseda et al., 2004). Partly because of the acquisition of heteronomous DNA fragments, the AT content along the genome is not

equally repartitioned, with regions as long as tens of kilobases having a higher average AT content than surrounding sequences (Eisen, 2000; Lawrence and Ochman, 1997)(H-NS Fig. 6).

Three different levels of H-NS dependent regulation as a function of chromosomal position

We found that Phns promoter expression in the LT position is always more repressed by H-NS than the other sites, such as RT, which is equidistant from the origin. The reporter at the LT position, and its neighboring insertions, LT1 and LT2, are situated in a ~10 Kb region with an AT content higher than average (H-NS Fig. 5 and SI Fig. 4). The repression in LT is probably enhanced by the fact that it is found near the boundary with the left macrodomain, featuring a significant cluster of horizontally acquired AT-rich genes and silent pseudogenes (Zarei et al., 2013). An additional factor that could facilitate DNA compaction by H-NS and stabilize H-NS oligomerisation at the ter macrodomain is the presence of binding sequences for the MatP protein, which has been shown to play a structuring role by the formation of bridges between different sites (Dupaigne et al., 2012; Mercier et al., 2008).

The greatest difference between LT and RT sites is observed when looking at the growth rate dependence of expression. LT is significantly more repressed than RT at slower growth rates, resulting in a constant YFP concentration as a function of doubling time (H-NS Fig. 5). This indicates that H-NS activity is higher at the LT site at slow growth. A similar effect is observed comparing RO and LO, but to a smaller extent (H-NS Fig. 3). The constant YFP concentration (YFP/OD equivalent to YFP/mass) as a function of growth rate of the LT strain is consistent with what would be expected from a gene cooperatively repressed by negative auto-regulation (Klumpp et al., 2009). The hns gene is found in an environment similar to the one of LT (SI Fig. 4), therefore this suggests that H-NS itself may follow a similar pattern of expression. At slower growth there is a reduced dilution rate and a smaller amount of DNA per cell, and thus less H-NS binding sites per cell. This could result in a greater amount of H-NS available to bind a promoter and repress gene expression, particularly in the case of decreasing amounts of FIS (see below). The other strains are less sensitive to H-NS repression in exponential phase. Variation of their YFP concentration as a function of growth rate is similar to that expected from either a weakly repressed or a constitutive promoter, whose gene product becomes more diluted at fast growth, independently of the gene copy number.

A different pattern in the level of expression is observed when growth rate slows down during entry into stationary phase. During this growth phase transition the YFP concentration from RO is significantly lower than the one measured in LO. Similarly, in these conditions the expression from RT1 is lower than the one from RT. An important factor contributing to the difference in expression among the strains can be found in the interplay between the NAPs along the bacterial growth curve. In the literature there is ample evidence for changes in the composition of NAPs according to growth phase and to growth rate of the cell(Ali Azam et al., 1999; Browning et al., 2010; Dillon and Dorman, 2010; Ishihama, 1999; Ohniwa et al., 2006). FIS, a NAP known for being necessary for fast growth (Nilsson et al., 1992a) and for being involved in shaping the chromosome (Dame, 2005; Schneider et al., 2001), is expressed in a growth rate and growth phase dependent fashion. A peak of the cellular FIS concentration is observed in exponential phase, while it becomes undetectable in stationary phase (Ali Azam et al., 1999; Nilsson et al., 1992a). In exponential phase the higher FIS concentration can better compete with H-NS at the Phns promoter (Falconi et al., 1996), explaining the lack of difference in expression between the sites during this growth phase.

The competition between FIS activation and H-NS repression occurs also at other genes, such as the ribosomal promoters (Afflerbach et al., 1998; Nilsson et al., 1992b; Schröder and Wagner, 2000). Furthermore, in similar growth conditions as those used here, ChIP assays have shown that there is a significant overlap between FIS binding and AT-rich regions and FIS and H-NS binding to the genome(Cho et al., 2008; Grainger et al., 2006). Therefore when the amount of FIS in the cell decreases at the end of exponential phase, H-NS can extend its action on the chromosome and to a higher extent in the regions with a higher amount of H-NS binding sites. Our data support this idea: the expression from LO and RO is similar when FIS is abundant, and lower in RO when FIS concentration decreases, due to the presence of AT-rich H-NS binding sites near RO. The same effect can be seen in the difference between RT and RT1 at entry into stationary phase.

Recent results obtained on purified nucleoids have shown that H-NS plays an important role in maintaining the level of compaction of the nucleoid when the total level of transcription activity decreases during the transition to stationary phase (Thacker et al., 2014). Previous studies have shown that ongoing transcriptional activity can contribute to increased compaction of the nucleoid (Cabrera et al., 2009; Jin et al., 2013). Most of the transcriptional activity in the cell derives from ribosomal genes that are activated by FIS in exponential phase and repressed by H-NS (Afflerbach et

al., 1998; Schröder and Wagner, 2000). An increase in H-NS to DNA ratio upon entry into stationary phase could thus explain at the same time both the decrease in total transcription activity and the compensatory compaction of the nucleoid. This H-NS dependent repression however takes place first at the genes within AT-rich regions of the nucleoid during the transition to stationary phase and then is extended to the other genes as cell growth slows down even more in stationary phase.

In a parallel work, similar reporter constructs using the fis and dps genes promoters were inserted in some of the same positions used here (Berger et al., 2014). Both of these reporter constructs, showed a chromosomal position dependent expression, but only in a hupA/B- background, in which the global nucleoid structure is altered (Jaffe et al., 1997). For the dps promoter a chromosomal position dependent expression was observed when the insertion was placed at the LT position or within a specific genomic island in the ABU8379 strain, known for being AT-rich and for containing non-essential genes (Hacker and Kaper, 2000). The dps promoter is also regulated by both H-NS and FIS, albeit by a different mechanism compared to the Phns promoter that results in its induction in late stationary phase (Grainger et al., 2008). These results therefore show that H-NS regulation is necessary but not sufficient to determine a chromosomal position dependence of gene expression and that this positional effect is also dependent on the promoter sequence used.

Recently a similar analysis of gene expression as a function of chromosomal position has been presented by Block and coworkers (Block et al., 2012) and Ying and coworkers (Ying et al., 2014). They inserted in different positions of the chromosome the gene for a fluorescent reporter protein under the control of a synthetic promoter, PLacO-1 or Ptet respectively. The first is an inducible promoter repressed by LacI, while the second is repressed by TetR. In agreement with previous results obtained in E. coli and other bacterial species (Chandler and Pritchard, 1975; Dryselius et al., 2008; Schmid and Roth, 1987; Sousa et al., 1997; Thompson and Gasson, 2001), no differences in expression as a function of chromosomal position, gene orientation and regulator-target gene distance was detected. Here again, the reason for the differences between our results and the previous ones could lie in the different promoters used, since the promoter used here is repressed specifically by H-NS, and that in this case we carried out measurements as a function of growth phase and growth rate while in most of the works cited above gene expression was measured in mid exponential phase in a single or at most two growth media. On the other hand, the result showing that the concentration of

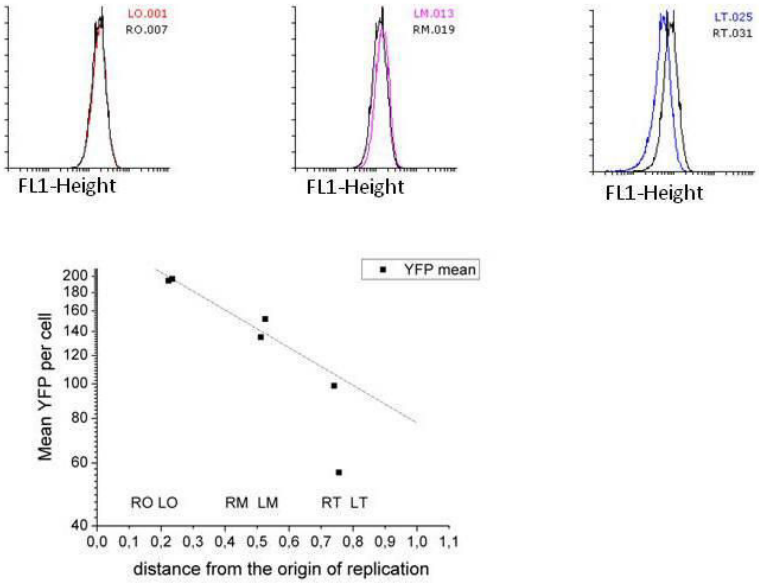
YFP expressed from Phns at the different chromosomal insertions doesn't depend on the distance from the regulator gene agrees with the data obtained previously.

6.1.6 Overall conclusions

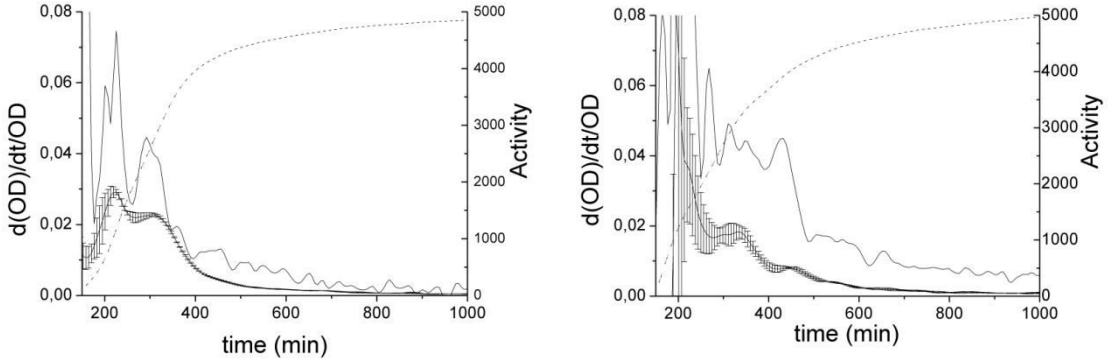
A number of cellular parameters affecting the gene expression change during both growth transitions and cellular adaptation. These include the increased dilution rate of transcription factors due to the cell division and the amount of DNA per cell due to overlapping DNA replication rounds; the amount of active ribosomes and of available RNA polymerase, taking into account changes in the amounts of the different sigma factors; the concentration of small metabolites, such as ppGpp and cAMP; the concentration of nucleoid proteins and the level of negative supercoiling (Berthoumieux et al., 2013; Klumpp and Hwa, 2014; Travers and Muskhelishvili, 2005b). This type of regulation can be thought of as an analog control, complementing the digital control, i.e. the network of regulation mediated by dedicated transcription factors (Blot et al., 2006; Marr et al., 2008; Sonnenschein et al., 2011). All of these factors can potentially affect gene expression independently of where the genes are found in the genome. The genomic sequence however, and especially its AT content, can affect both the affinity for nucleoid proteins and the stability of the DNA under torsional stress due to changes in topology (Sobetzko et al., 2013).

Here we provide evidence for modulation of gene expression depending on the chromosomal position by a global regulator. We have identified three different levels of regulation: those regions where H-NS has small effect (RT, LO, RM, LM); regions where regulation by H-NS is conditional (RT1 and RO) and a region where H-NS repression is strongest and results in a growth rate independent protein concentration (LT and its neighbors, LT1 and LT2). This indicates that the position of H-NS regulated genes will influence their growth phase, growth rate and temperature dependence of expression. The nucleoid protein dependent structure of the chromosome can thus affect the gene expression in *E. coli.*

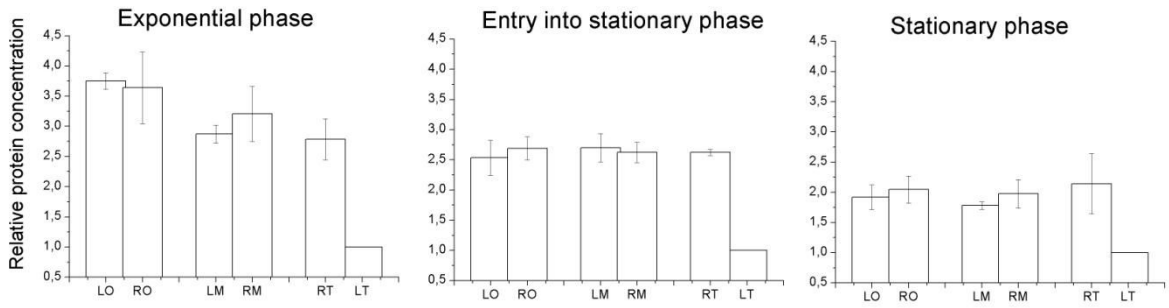
6.1.7 Supplementary information



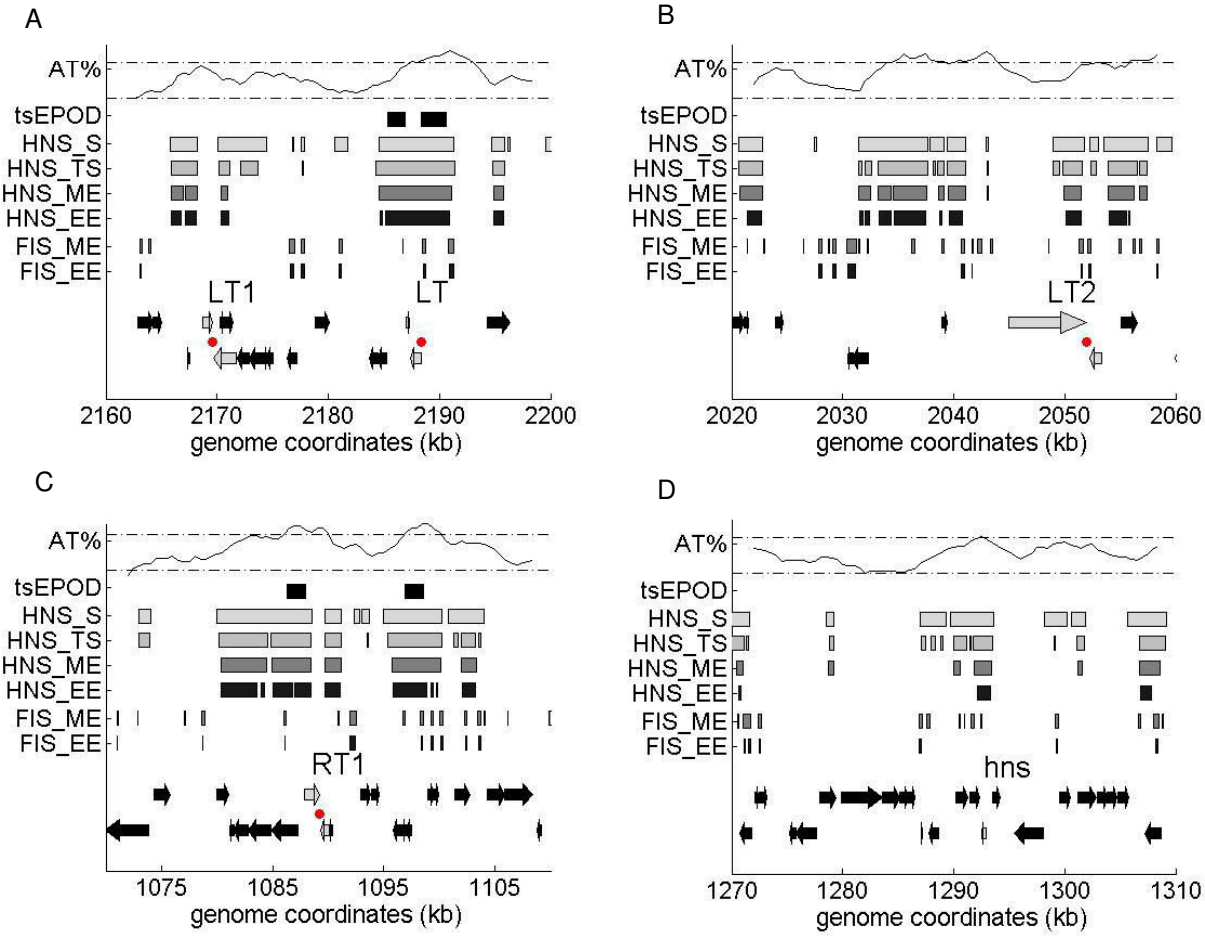
SI Figure 1 Fluorescence intensity within the bacterial population is homogeneous. Example of FACS measurement of the six bacterial strains. The distribution of YFP per cell is normal for all the strains, excluding therefore heterogeneity in the population. In the plot comparing LT and RT it is possible to notice how the LT strain (blue) is less fluorescent than the RT strain (black). This is more evident when plotting the mean of the YFP distribution as a function of the distance from the origin of replication. The mean YFP for the LT strain is lower than that expected from the difference in gene copy number (dotted line).



SI Figure 2 The increase in YFP concentration in stationary phase at slow growth is due to the delay in the decrease of promoter activity with respect to the decrease in growth rate. The change in OD is shown by a dashed line, the change in growth rate (d(OD)/dt/OD) in the line with the error bars from the three technical repeats within the experiment and the change in promoter activity (d(YFP)/dt/OD) by a continuous line. (A) Growth rate and promoter activity for cells growing in M9 minimal media supplemented with 0.4% glucose and 0.5% casamino acids (fast growth). The decrease in growth rate at the entry into stationary phase happens at the same time as the decrease in activity, leading to no accumulation of YFP (H-NS Fig. 2A main text). (B) Growth rate and promoter activity for cells growing in M9 minimal media supplemented with 0.2% casamino acids (slow growth). The decrease in activity happens later than the decrease in growth rate, there is therefore an accumulation of YFP at the entry into stationary phase for cells growing more slowly.

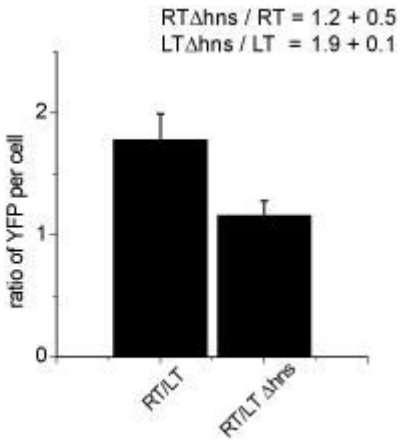


SI Figure 3 No difference in YFP concentration between RO and LO as a function of growth phase at 37°C compared to 30°C (H-NS Fig. 4 in the main text). YFP concentration was normalized by the LT values for strains in exponential, entry into stationary and stationary phase for three independent experiments, the error bars indicate the SEM. Data were taken at the time of maximum growth rate, at the time where the growth rate was half of the maximum and at growth rate equal to zero, respectively. The YFP concentration in the LT strain is always lower than in the other strains. AT 37°C there is no significant difference between LO and RO.

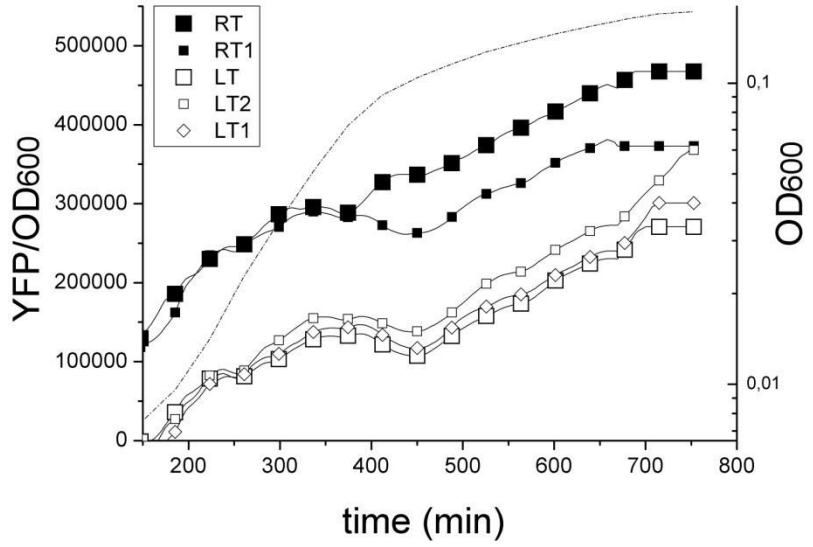


SI Figure 4 Genomic neighborhood of the additional insertions near LT and RT and of the *hns* gene shows that these sites are found in regions with a higher than average AT content and large H-NS bound regions that

increase in size as the cells enter stationary phase. For each plot, from the bottom to the top: Genes on the lagging and on the leading strands. In grey are plotted the convergent genes where the insertions have been made, and the *hns* gene itself. FIS binding in early exponential phase (FIS_EE) and in mid-exponential phase (FIS_ME), data from (Kahramanoglou et al., 2011). Sites bound by H-NS in early exponential (HNS_EE), mid-exponential (HNS_ME), transition to stationary (HNS_TS) and stationary (HNS_S) phases (Kahramanoglou et al., 2011). On the top of these, in LT and in RT1 there are also tsEPOD (Vora et al., 2009). AT content is calculated within a 4000 bp sliding window with a shift of 500 bases. In the proximity of all the insertions it is possible to detect a strong occupancy by H-NS as well as a higher AT content of the genomic sequence. For the *hns* gene there is a clear increase in the length of the H-NS bound region while cells are passing from exponential to stationary phase.



SI Figure 5 The difference between RT and LT is lost in a Δ*hns* background. The bar graph shows the ratio between the average YFP per cell for the RT and LT insertions in a WT background and Δ*hns* background. Data were obtained by flow cytometry for cells in exponential phase growing in 0.5% casamino acids at 37°C in flasks, shaking. Error bars represent the standard deviation for four independent experiments. The ratio between the fluorescence in RT and LT becomes closer to 1 in the Δ*hns* background with respect to the ratio in the wild type background. This is due to a stronger increase in fluorescence for the LT position in absence of H-NS with respect to the slight increase of fluorescence for RT position in the Δ*hns* background.



SI Figure 6 P*hns* promoter activity remains similar for insertions placed up to 135 Kb away from the original sites. Plate reader experiment for strains growing in M9 supplemented with 0.2% casamino acids, at 37°C. The effect of silencing in the LT position extends over several tens of kilobases (LT position= 2185402, LT2=2050038 (Δ135364), LT1=2167635 (Δ17767)), such that the signal from the neighboring strains LT1 and LT2 is similar to the one in LT strain. The YFP concentration in RT1 is similar to the one in RT (RT=1027582, RT1=1093457 (Δ65875)) during exponential phase, while at the entry into stationary phase the expression of RT1 is lower than that of RT.

6.2 How does chromosomal position influence the expression of a supercoiling-sensitive ribosomal promoter?

In the previous chapter I showed that the chromosomal position affects the expression of a complex promoter such as the *hns* promoter. This modulation of gene expression can be ascribed to the different level of protein binding, notably H-NS itself, in the different parts of the chromosome, as a function of the AT content. But are there other aspects of chromosomal organization that can influence gene expression? In order to answer this question, we inserted in the chromosome the ribosomal promoter *rrnBP1* (a shortened version without the FIS binding sites, see below) and the T5 phage *P5* promoter driving the expression of the GFP reporter gene. The P1 promoter is sensitive to supercoiling and ppGpp, while P5 is a strong constitutive promoter (see 5.3 and below for a detailed description). In Figure 15 the sequences of the two promoters are compared: both promoters have the AT-rich UP element, required for recruiting the polymerase via an interaction with the α-CTD (Ross and Gourse, 2005). P5 has consensus -35 and -10 sequences and the optimal 17bp-spacer, while P1 has a suboptimal -35 sequence and a suboptimal 16bp-spacer, both "sub-optimalities" necessary for growth rate control (Josaitis et al., 1995), a consensus -10 and the GC-rich discriminator region overlapping the transcription initiation site also necessary for the growth rate control because it makes P1 promoter sensitive to supercoiling (Travers, 1976). One aspect that could change as a function of chromosomal position is the level of supercoiling, and P1 could act as a supercoiling sensor due to the presence of the discriminator in the promoter region, while P5 is less sensitive to supercoiling and therefore can be taken into account as a control. This version of the P1 promoter, lacking the FIS binding sites, in addition should be more sensitive to changes in DNA topology given the role of FIS in stabilizing RNAP in the absence of negative supercoiling (Pemberton et al., 2002). We therefore studied P1 and P5 expression as a function of chromosomal position at different growth rates and in different growth phases. We also studied the effect on the chromosomal reporter activity of an upshift in growth rate, and measured the change in gene expression as a function of neighboring genes and upon inhibition of translation, influencing the cellular ppGpp concentration. The results I will present in the following paragraphs have been obtained by me in collaboration with Damel Mektepbayeva, Master 2 student, and Qing Zhang, postdoc.

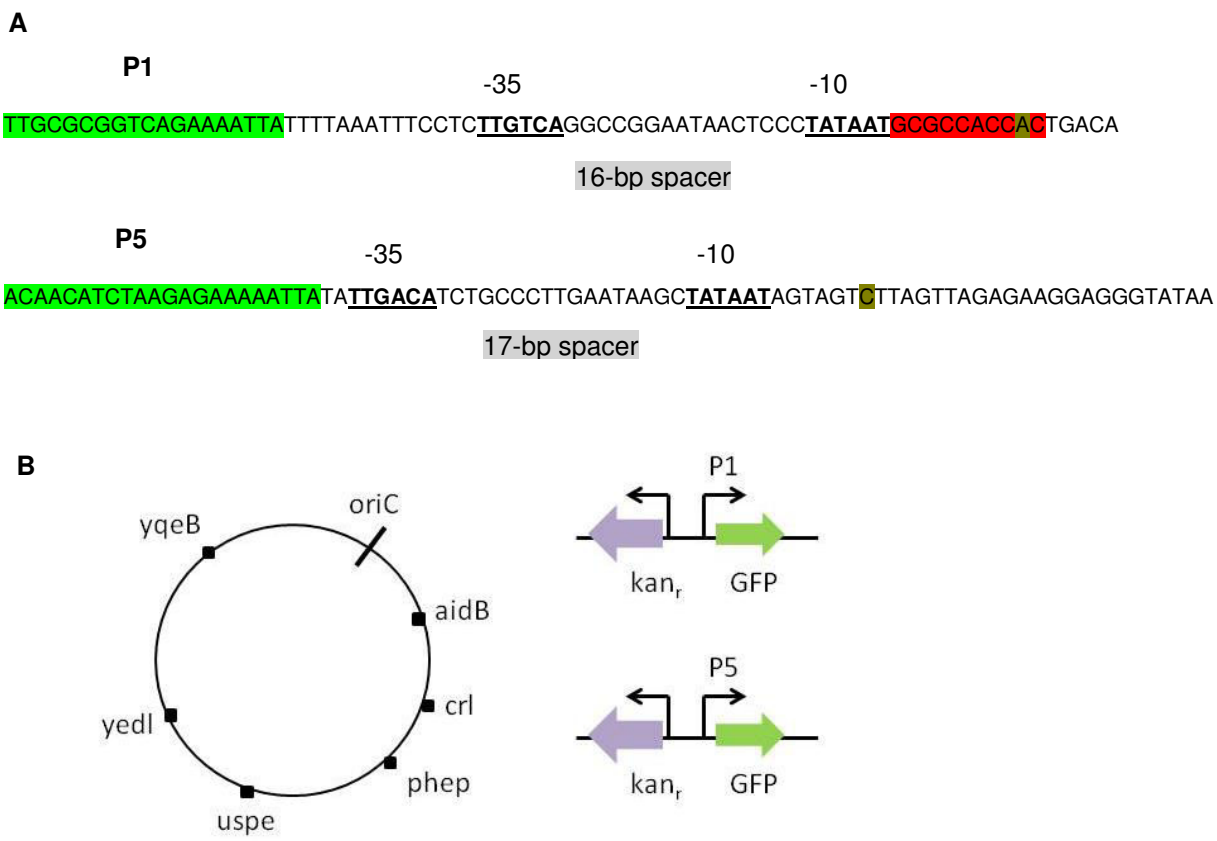
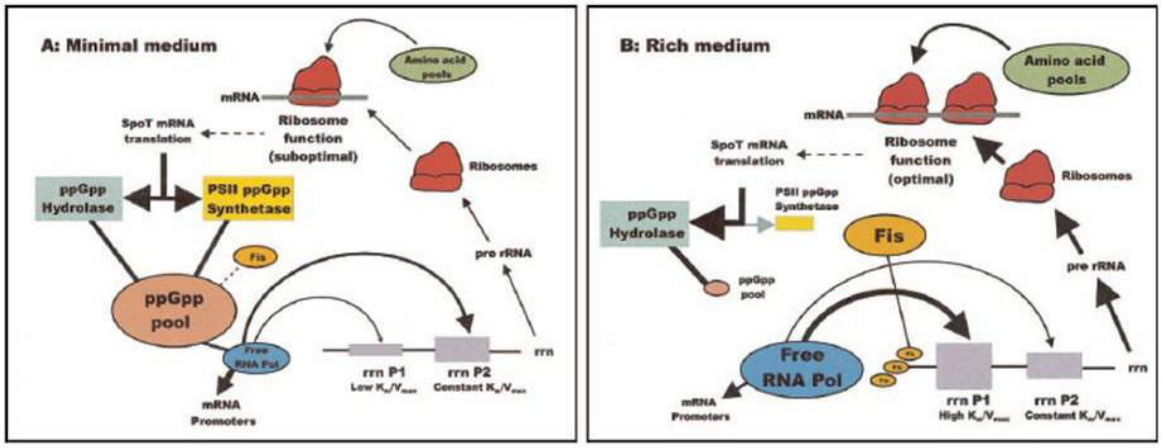


Figure 15 A) The sequence of the core rrnBP1 promoter and the one of the T5 phage P5 promoter. In light green the UP element, underlined the -35 and -10 regions, between them the spacer, in red for P1 the discriminator region, in dark yellow the transcription start site. B) A scheme of the two construct promoter+GFP+kanamicyn gene and the positions in which they have been inserted in the *E. coli* chromosome.

6.2.1 P1 and P5 expression as a function of chromosomal position at different growth rates

As I reported before, ribosome synthesis is tightly regulated in order to respond to change in growth rate (Schaechter et al., 1958), both by supercoiling level and by ppGpp. While ppGpp diffuses rapidly in the cell, and therefore the concentration of ppGpp should be the same along the whole chromosome, it is possible that the level of chromosomal supercoiling can vary. As I already described in the introduction, several factors that are known to affect supercoiling, such as NAPs binding sites or RNAP foci, are not evenly distributed along the chromosome (Grainger et al., 2005, 2006; Prieto et al., 2012; Sobetzko et al., 2012; Zarei et al., 2013). It is therefore interesting to test if a change in the chromosomal position affects the precise regulation acting on the ribosomal promoter, with P5 promoter as a control.

Box 5 Ribosome synthesis regulation



(Dennis et al., 2004)

Ribosomes are made of both proteins and rRNA. The ribosomal proteins are negatively autoregulated, when there is not enough rRNA to bind, they bind to their own mRNA, preventing translation (Liang et al., 1999b; Mattheakis et al., 1989). There are seven rRNA operons in *E. coli* (Ellwood and Nomura, 1982) with a similar level of expression once corrected for gene copy number (Condon et al., 1992), and each of them is expressed by two promoters, P1 and P2. P2 is a constitutive promoter and maintains a basal level of expression proportional to the amount of free RNAP (Dennis et al., 2004), while P1 is sensitive to supercoiling (Oostra et al., 1981) and growth rate, due to the presence of the discriminator region bordering the -10 sequence (Josaitis et al., 1995). P1 expression is enhanced by FIS (Muskhelishvili et al., 1995, 1997) and repressed by the alarmone ppGpp (Condon et al., 1995; Zacharias et al., 1989). FIS does not affect stringent control or growth rate regulation of ribosomal promoters (Ross et al., 1990), but it is necessary in order to sustain P1 activity at fast growth (Dennis et al., 2004), while during slow growth ppGpp prevents FIS activation of P1 (Nilsson et al., 1992b).

With this aim, we measured the change in fluorescence in the different strains as a function of growth rate. As for the P*hns* strains, the overnight cultures were diluted 1:10000 in different growth media and then grown at 37°C in the plate reader, shaking. OD_{600} and fluorescence were measured every 5 minutes, and the protein concentration as a function of time was calculated for the different strains. Figure 16 shows the values for protein concentration in exponential phase as a function of growth rate for the different P1 and P5 chromosomal insertions. At increasing growth rate the protein concentration for the ribosomal promoter P1 increases, as expected. P5 shows an opposite behavior, with accumulation of the protein at slow growth rate and dilution of the protein at fast growth. This is consistent with the behavior of a constitutive promoter as previously described (Klumpp et al., 2009).

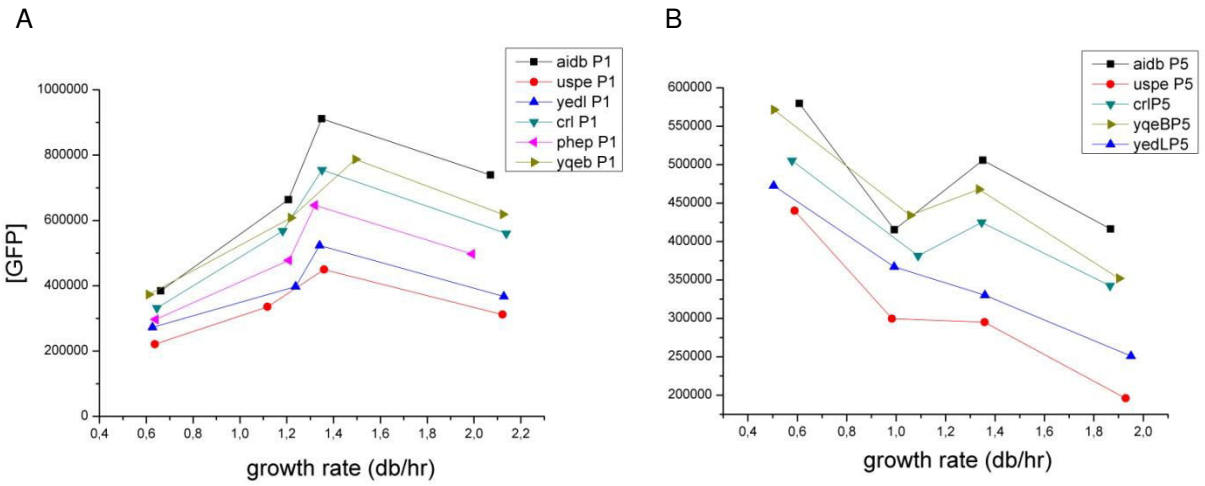
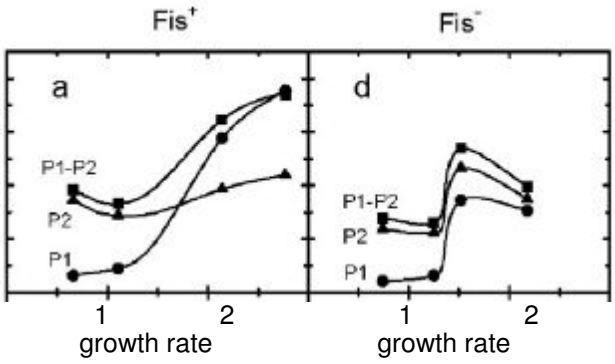


Figure 16 GFP concentration as a function of growth rate for the P1 (A) and P5 strains (B). For the ribosomal promoter there is an increase in protein concentration at increasing growth rate, while expression of the P5 promoter decreases at fast growth rate. All the different chromosomal insertions show a similar dependence on the growth rate.

The decrease in GFP concentration for the P1 strains at fast growth (more than 1.5 db/hr) is due to the fact that, with respect to the original *rrnBP1* promoter sequence, the one we used here lacks the FIS binding sites, which are required for activation (Zhang and Bremer, 1996). This behavior is in agreement with what has been previously described (Dennis et al., 2004), as shown in Figure 17.



(Dennis et al., 2004)

Figure 17 Figure from Dennis et al showing β-galactosidase activity as a function of growth rate expressed from rrnBP1, P2, and associated P1-P2 promoters in the wild-type and ΔFIS strains as a function of growth rate. FIS is necessary in order to ensure high promoter expression at fast growth.

Both for P1 and P5 the trend of protein concentration as a function of growth rate is similar for all the chromosomal positions, with the insertions close to the origin (aidb and yqeb) showing a higher protein concentration than the one close to the terminus (uspe, yedl). In order to verify whether this difference in the level of protein expression is a result of a difference in gene copy number, we normalized

protein concentration by dividing for the gene copy number expected at the given growth rate. The result in Figure 18 shows in fact that the observed differences in protein concentration at the different chromosomal positions can be explained by the differences in gene copy number.

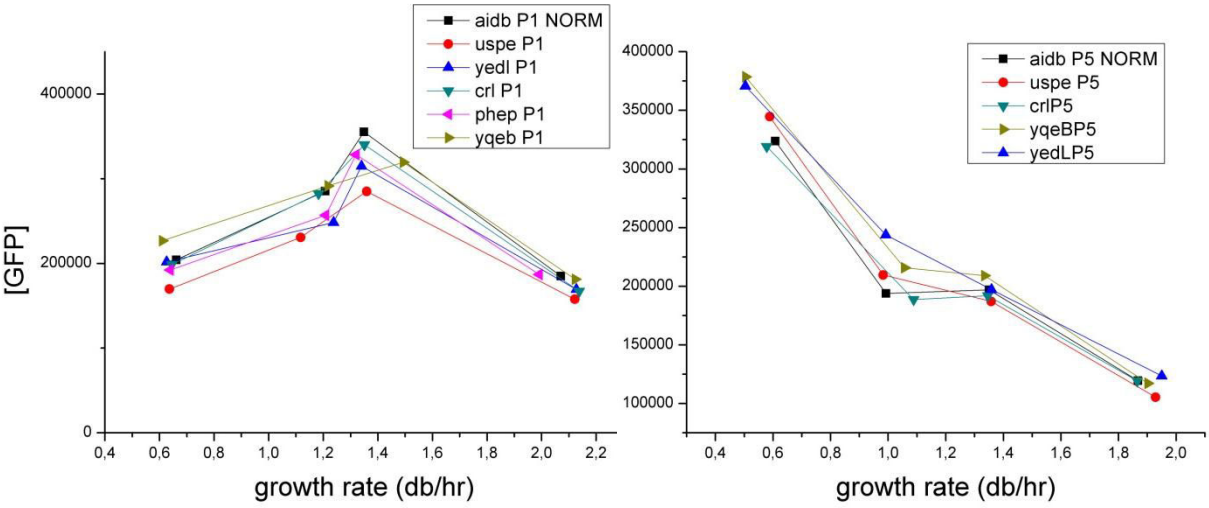


Figure 18 Protein concentration as a function of growth rate for P1 and P5 strains normalized by the gene copy number. Gene copy number was calculated from the Cooper-Helmstetter equation. It is possible to notice that the differences among different chromosomal position vanish after normalization.

6.2.2 P1 and P5 expression as a function of chromosomal position at different growth phases

Since changes in growth phase are accompanied by changes in supercoiling level and ppGpp (Balke and Gralla, 1987), we measured the expression of the reporter promoters P1 and P5 in the different chromosomal positions in order to test whether the different parts of the chromosome are differently affected by growth phase transitions. Figure 19 shows that the GFP concentration for the P1 promoter decreases while the cells move toward stationary phase independently of the position on the chromosome and on the growth medium, while the concentration for P5 increases. Again, this result agrees with the expected change in ppGpp concentration resulting in a decrease in P1 promoter activity at entry into stationary phase. On the other hand, since the P5 promoter is constitutively expressed, its GFP protein accumulates in the cell when the growth rate decreases due to a faster

decrease in the dilution rate compared to the change in promoter activity. This difference in activity between P1 and P5 as a function of growth phase is visualized in Figure 21.

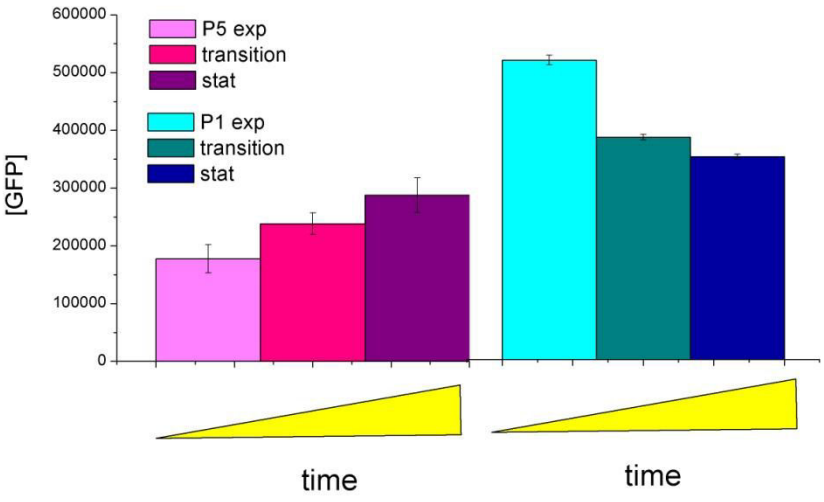


Figure 19 Protein concentration for P5 and P1 promoter inserted in the Crl position growing in M9+glu04caa05 at 37°C. While the cells move toward stationary phase there is an increase in GFP concentration for the P5 strain and a decrease in GFP concentration for the P1 strain, due to the different regulation acting on them. The error bar is given from the average of two independent experiments.

If we consider the dependence on the growth phase for the different chromosomal positions for cells growing in different growth media, we obtain a different behavior for P1 and P5 (see Figure 20). In order to compare the different datasets, the value for protein concentration in the uspE strain has been set equal to one.

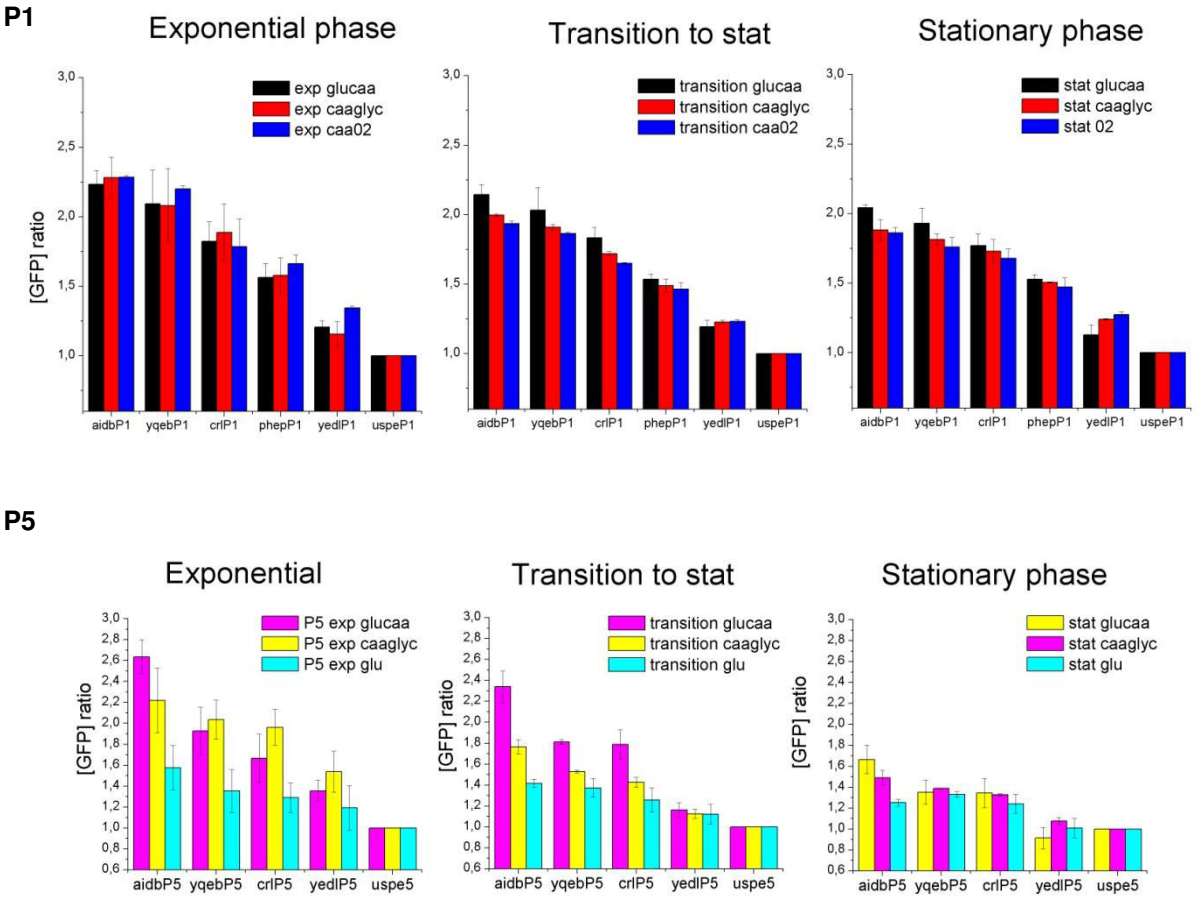


Figure 20 Protein concentration for the P1 and P5 promoters in the different chromosomal position (x axis) in three different growth media (colors, richer medium on the left), normalized on the uspe value. While the cells move toward stationary phase, P1 expression stops in all positions (Figure 21), therefore the protein concentration is diluted to the same extent in all the strains, leaving the ratio between them unchanged. For P5, in exponential phase there is a clear effect of gene copy number on GFP concentration, with the insertion near the origin producing more GFP than the one near the terminus. This difference is lost once replication is arrested, because the promoter is still expressed and therefore the GFP concentration in the different strains becomes similar.

As expected from the changes in gene copy number, in both the P1 and P5 strains the GFP concentration is higher near the origin than in the terminus. For the P5 promoter this difference decreases in stationary phase, coherently with a slowdown of replication and the consequent decrease of the origin/terminus ratio. For the P1 promoter the difference in protein concentration between the origin and terminus insertions remains similar as the cells enter into stationary phase. This can be explained by the P1 promoter being shut off in all the chromosomal positions before the entry into stationary phase, resulting in the same dilution of the GFP for the different strains and therefore maintaining the difference between origin and terminus. The promoter activity decreases abruptly for P1 and more smoothly for P5 when the growth rate decreases in the growth curve (shown

in Figure 21). This rapid decrease can be attributed to the stringent response acting on ribosomal promoters (see chapter 2.4). The two different peaks in P1 activity reflect the existence of two growth rates during bacterial growth in M9+caa02 (the growth rate as a function of time is displayed in black in Figure 21). The higher growth rate corresponds as expected to higher ribosomal promoter activity. When ribosomal promoter expression is repressed during the stringent response, there is an increase in the amount of polymerase available for transcribing other promoters (Zhou and Jin, 1998). We don't detect an effect of this passive regulation on P5 activity because at entry into stationary phase there is also an increase in the sigma factor σ^s (Loewen et al., 1993). This sigma factor competes with σ^{70} (Farewell et al., 1998; Mauri and Klumpp, 2014), resulting in a down regulation of σ^{70} promoters, such as P5 (see chapter 5.3),

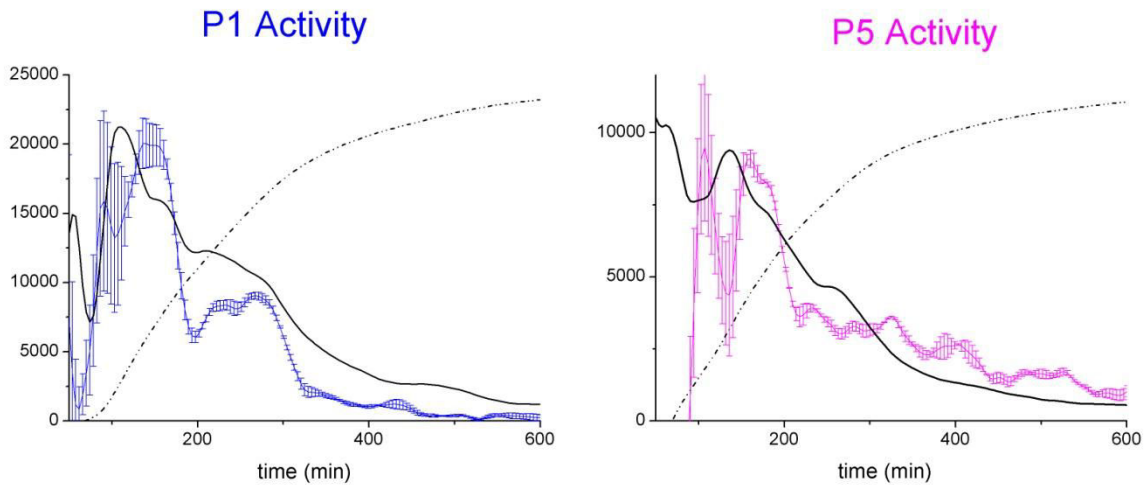


Figure 21 Promoter activity for P1 (blue) and P5 (pink) promoters inserted in the crl position and growing in M9+CAA02 at 37°C. The growth rate is in black, while the OD curve is dotted. P1 activity stops abruptly at the entry into stationary phase (300 minutes), while P5 activity decreases more gradually.

We have been able to identify a specific dependence on growth phase of GFP concentration for the P5 and P1 promoters, but we could not identify a specific influence of chromosomal position on promoter expression. In order to point out possible differences among the chromosomal insertions, we measured gene expression for cells growing under stressed condition (inhibition of translation) and for cells adaptation after a shift from one growth media to another. The results are presented in the next paragraphs.

6.2.3 Changes in P1 and P5 expression when translation is inhibited

As I previously described, by using sublethal concentration of chloramphenicol one can observe cell growth and promoter activity upon inhibition of translation (Wolfe and Hahn, 1965). This inhibition results in an increase of the internal concentration of amino acids (Kurland and Maaløe, 1962) and in a consequent decrease of ppGpp (Gallant et al., 1972) and a derepression of ribosomal promoters in order to have enough ribosomes to continue cell growth even at decreased ribosomal activity (see chapter 3). Therefore in the presence of chloramphenicol, a large part of RNAP and ribosomes are allocated for ribosome synthesis. As it was suggested by Scott et al., inhibition of translation results in an increase of the fraction of transcription and translation resources devoted to the expression of ribosomal genes and synthesis of ribosomal proteins at the expense of the metabolic proteins expression (Scott et al., 2010).

This enhanced synthesis of ribosomes compared to the rest of metabolic proteins could induce changes in the distribution of RNAP and ribosomes in the cell, concentrating them closer to the origin where the ribosomal operons are located. If such an effect existed, we could detect it by measuring the expression of our reporters in conditions of translation inhibition. From what is described above, we expected that in presence of chloramphenicol P1 expression should increase, while P5 expression should decrease (see Figure 9).Surprisingly, when we measured GFP expression for bacteria growing with different concentration of chloramphenicol we detected an increase in the fluorescence for both theP1 and P5 strains as a function of increasing chloramphenicol concentration (see Figure 22).

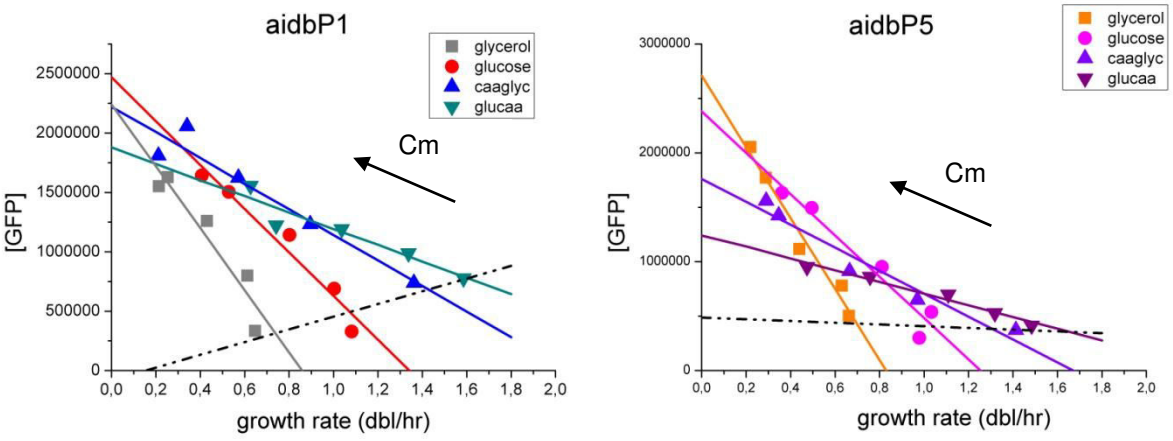


Figure 22 Protein concentration as a function of growth rate in four different media (colors) at different chloramphenicol concentrations (lower concentration on the right, increasing toward the left) for the aidb insertion of P1 and P5 promoter. The GFP concentration for both promoters increases at increasing chloramphenicol concentration.

This result does not agree with the theory by Scott et al., therefore we tried to understand what was different between their experiment and ours. In their experiment they measured the β-gal activity per unit of total protein. In our experiment we measure the fluorescence per unit of OD_{600}, i.e. per mass. It has been shown that the protein per mass remains fairly constant as a function of growth rate (HANS BREMER, 1996), therefore our measurements should be compatible to theirs.

We asked ourselves whether the difference between our assay and theirs could reside in the rate of translation of the reporter gene. The codon usage could differ between our reporter (*GFPmut2*) and their reporter (*lacZ*). It was shown that ribosomal and highly expressed proteins in general use codons with high translation rate (Gouy and Gautier, 1982; Post et al., 1979; Sharp and Li, 1986), and that mRNA with infrequent codons required a longer translation time than mRNA with 'fast codons' (Sørensen et al., 1989). This delay could be amplified by the inhibition of translation, giving an advantage to translation of mRNA with fast codons. We used the classification of optimal codons suggested by Klumpp et al. in order to calculate the percentage of fast codon in the reporters sequences (Klumpp et al., 2012). The results are shown in table 3. The *GFP_mut2* gene has a lower percentage of fast codons than *lacZ* and a lower percentage than other metabolic, regulatory and ribosomal genes in *E. coli*. On the other hand, the *GFP_mut2* sequence is shorter than *lacZ*, resulting in a faster synthesis rate. Could this difference in length in conditions of translational inhibition matters? In order to test this hypothesis, we should either study P5 expression associated with lacZ, either use simulation in order to assess the effect of having a promoter with high transcription rate associated with a short reporter compared with a promoter with low transcription rate and a long reporter.

Gene	Number of codons	Percentage fast codons
GFP_mut2	238	46
lacZ	1025	58
dnaA	468	60
fis	99	72
fliA	240	58
hns	138	77
malP	798	65
rmsD	199	58

Table 3 Gene length and percentage of optimal codons (as defined in (Klumpp et al., 2012)) for our reporter, Hwa's reporter and several genes in *E. coli*. Important regulatory genes such as *hns*, *fis* or the ribosomal *rmsD* are short and rich in optimal codons, while the two metabolic genes taken into account are longer and with a lower percentage of optimal codons.

Between our and their measurements there is also a difference in the promoter considered. In their measurements of constitutive promoter expression, Scott et al. used a strain in which *lacY* gene was deleted, and in which *lacI* gene and the original *lac* promoter were replaced by the kanamycin resistance gene and the synthetic promoter P_{LtetO1} (Lutz and Bujard, 1997). The sequence of this promoter is compared to the one of P5 in Figure 23. Both promoters have a consensus -35, an optimal -17 bases spacer but P_{LtetO1} has a non-optimal -10. The degree of homology of the -10 region influences the rate of the isomerization step (Burr et al., 2000; Cook and deHaseth, 2007), while the UP element upstream the -35 region in P5 activates transcription by stabilizing the isomerization steps and quickening the transition from one intermediate to the following (Gourse et al., 2000). The strength of these two promoters can be measured with the method of Brewster et al. (Brewster et al., 2012), and this measurement shows an higher affinity for RNAP for P5 promoter with respect to the P_{LtetO1} promoter (see Figure 24). The P5 promoter is therefore stronger than the P_{LtetO1} promoter.

P_{LtetO1}

TCCCTATCAGTGATAGAGA**TTGACA**TCCCTATCAGTGATAGA**GATACT**GAGCACATCAGCAGGAC
GCACTGACCgaattcATTAAAG**AGGAG**AAAggtaccatg

P5

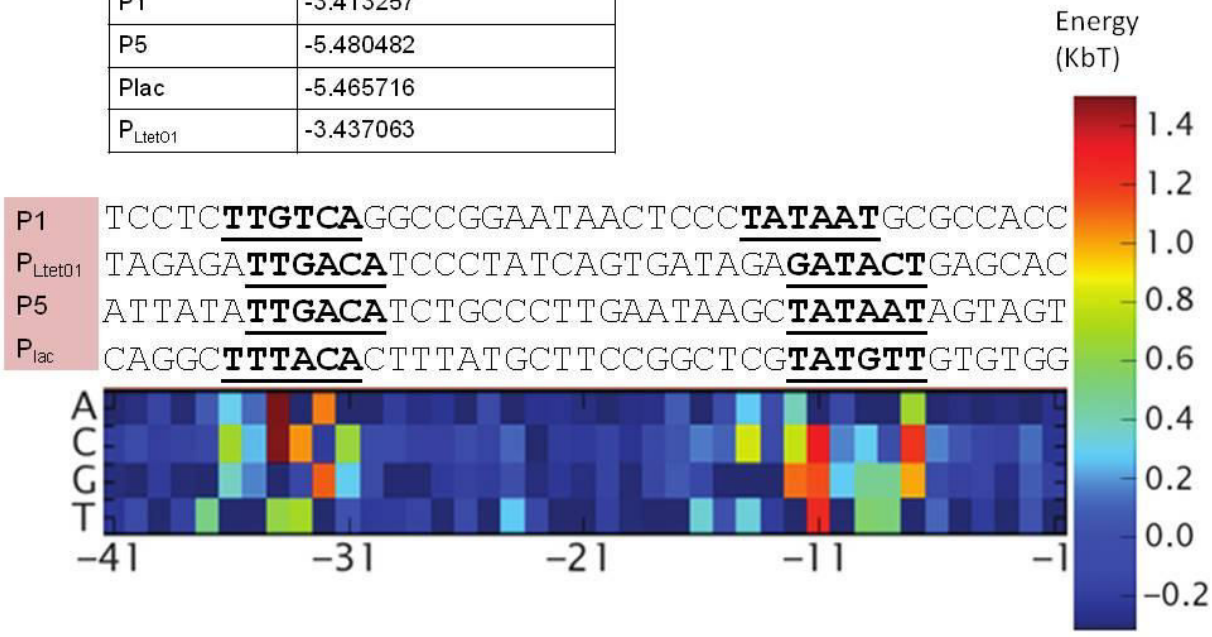
ACAACATCTAAGAGAAAAATTATA**TTGACA**TCTGCCCTTGAATAAGC**TATAAT**AGTAGTCTTAGTTA
GAGAAGGAGGGTATAATtctagaAATAATTTTGTTTAACTTTAAGA**AGGAG**ATATACAT**atg**

Plac

CCGATTCATTAATGCAGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACG
CAATTAATGTGAGTTAG**CTCACT**CATTAGGCACCCCAGGCTT**TACACT**TTATGCTTCCGGCTCGTA
TGTTGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTATGACCATGATTACGGA
TTCACTGGTCTAGAAATAATTTTGTTTAACTTTAAGA**AGGAG**ATATACATATGGCTAGCAAAGGAG
AAG

Figure 23 Promoter sequences for P_{LtetO1}, P5 and the version of Plac we use in our lab. -10 and -35 are underlined for P_{LtetO1}, P5 and for lacP2 promoter, while they are in grey for lacP1 promoter. The ribosome binding site is in yellow, the transcription start site in dark yellow and the restriction sites used for the cloning steps are in magenta.

promoter	Promoter strength (KbT)
P1	-3.413257
P5	-5.480482
Plac	-5.465716
P_{LtetO1}	-3.437063



Adapted from (Brewster et al., 2012)

Figure 24 Promoter strength calculated as in Brewster et al. The promoter strength was calculated by summing the energy contribution of the single bases in the promoter region. A low promoter strength indicates a high affinity for RNAP. This simple approach does not take into account the sequences upstream of -41, such as the UP element, or the effect of the discriminator region, therefore it can predict the activity of a specific subset of promoters, it is informative but not complete.

We decided to test whether the promoter sequence could be responsible for the increase in P5 expression at increasing chloramphenicol concentration, or whether this difference was to be ascribed to the short GFP gene. We therefore compared the expression of P5 to the expression of a weaker promoter associated with the same GFP reporter. We decided to use the *lac* promoter inserted in 3,080,693 bases position. Transcription initiation at this promoter is repressed by lacI (Gilbert and Müller-Hill, 1966) and activated by cAMP (de Crombrugghe et al., 1984) both directly and by inactivation the competing P2 promoter (Malan and McClure, 1984). Since lac promoter is weakly expressed (and therefore not suitable for the plate reader assay), we measured both the *lac* and the P5 strain using the flow cytometer for two different growth conditions and three chloramphenicol concentrations. For each condition, we measured GFP per cell and we normalized this value over the total amount of protein, stained with the AlexaF633H dye (Life Technologies). The result is shown in Figure 25.

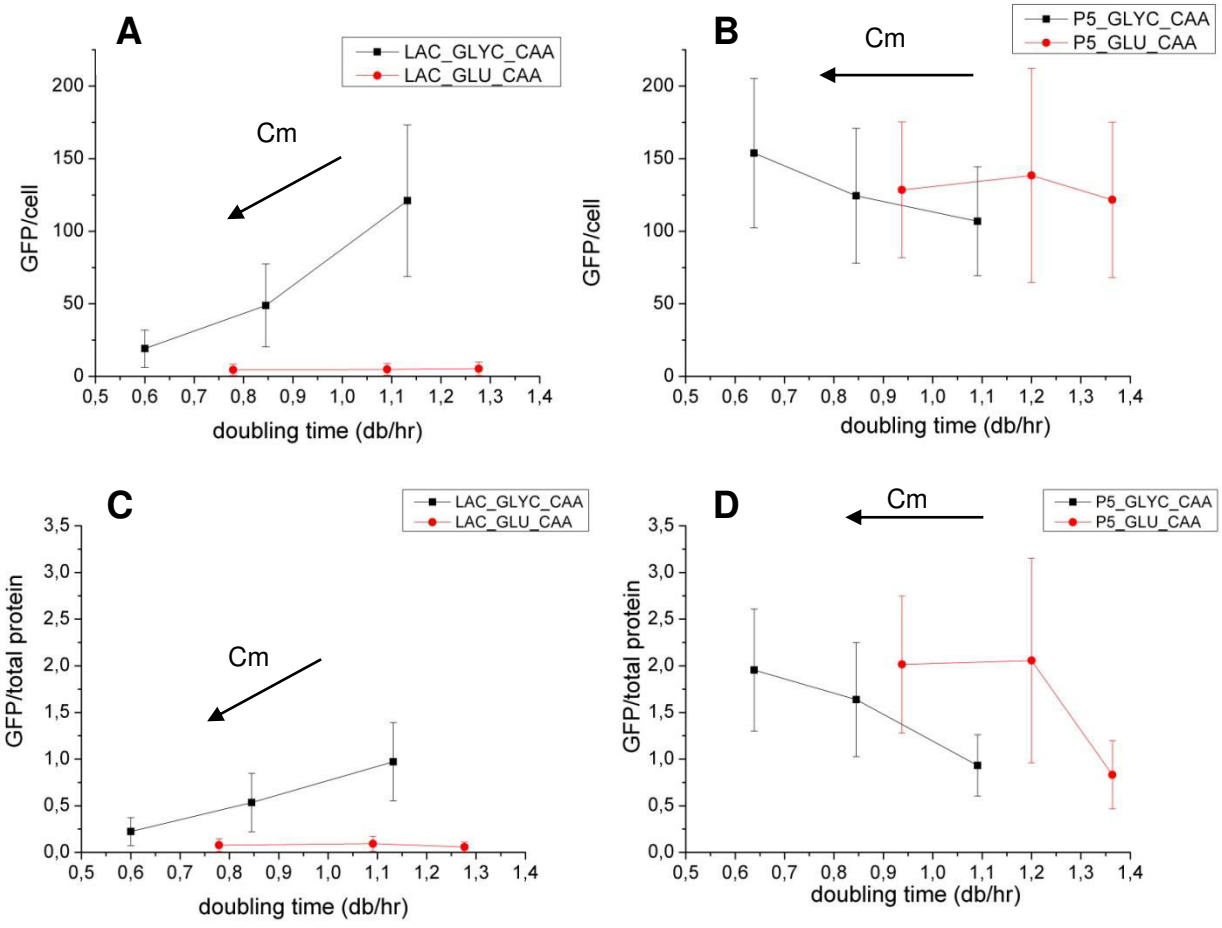


Figure 25 Flow cytometry assay for the Plac and P5 promoters in two different growth media supplemented with increasing amount of chloramphenicol. On the top, GFP per cell for Plac (A) and P5 (B) strains. On the bottom, the value of the GFP per cell is normalized over the total amount of proteins in the cell. The concentration of GFP for the weak Plac is almost zero in presence of glucose, and it is decreasing at increasing chloramphenicol concentration (C), while the concentration for P5 promoter is increasing with increasing chloramphenicol concentration (D).

As expected, in presence of glucose (red dots) the *lac* promoter expression is repressed. At increasing chloramphenicol concentration, the GFP expressed from the *lac* promoter decreases, both per cell and per total protein, while the protein expressed by P5 increases. This is in agreement with a study in literature in which it was shown that in the presence of chloramphenicol there was no transcription from the *lac* wild type promoter, while transcription was not stopped in the mutated promoter lacUV5 (Hirschel et al., 1980). We thus demonstrated with an independent experiment that our result for P5 is not an artifact resulting from the use of the GFP protein as a reporter of promoter activity, but it is a consequence of P5 strength.

There is a difference also in the distance from the origin of their insertion and ours, since our aidb insertion is located at 4,413,923 bases and their insertion is in the original *lac* position (365,529 bases). We then wanted to verify that the difference between the expected behavior of a constitutive and the P5 behavior was not due to our insertion being close to the origin of replication.

Knowing that mRNA translation is localized near the encoding gene (Montero Llopis et al., 2010), if the amount of free ribosomes in the cell decreases because ribosomes are mainly (and slowly) translating mRNA from ribosomal proteins, a gene located near the origin and therefore near the ribosomal genes could take an advantage with respect to a gene located near the terminus. For this reason we repeated the plate reader experiment with uspE insertion (1,395,696 bases).

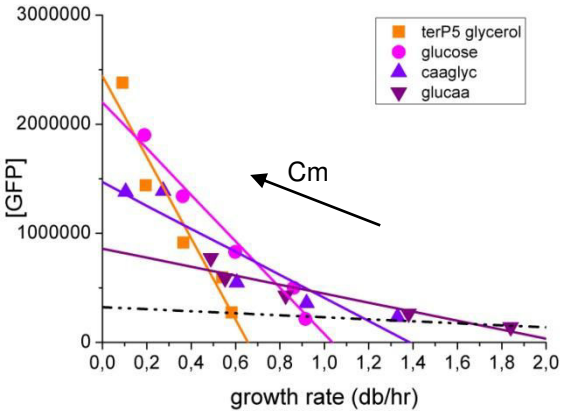


Figure 26. GFP concentration as a function of growth rate for P5 inserted in the uspe position in different growth media at different chloramphenicol concentrations. The increase in GFP at increasing chloramphenicol concentration is similar to what we measured for P5 inserted near the origin.

In the insertion near the terminus we found the same behavior of the P5 promoter inserted near the origin, with GFP concentration increasing at increasing chloramphenicol concentration, therefore the increase in protein concentration driven by P5 cannot be a result of a higher gene copy number in the aidb position we studied with respect to the original *lac* position of the Scott et al. study. We can positively affirm that the P5 promoter has a similar dependence on chloramphenicol as a ribosomal promoter. This common behavior can be explained by the similarity in the promoter sequence of P1 and P5: both are strong promoters, therefore they compete equally well for resources in conditions of translation limitation. However, P1 and P5 show differences for instance in the value of the intercept on the y axes, i.e. the maximal protein concentration attained when translational capacity is zero (see Figure 22 and table 4).

	P1		**P5**	
	intercept	slope	intercept	slope
glycerol	2,24E+06	-2,60E+06	2,71E+06	-3,26E+06
glucose	2,47E+06	-1,84E+06	2,38E+06	-1,90E+06
caaglyc	2,22E+06	-1,08E+06	1,76E+06	-1,05E+06
glucaa	1,88E+06	-685182	1,24E+06	-536014
no cm	-81481,7	535508,4	487424,2	-79496,2

Table 4 Values of the linear fit for GFP concentration as a function of growth rate in bacteria growing in one growth media with different chloramphenicol concentration.

For P1, the value of the Y intercept is weakly dependent on the growth medium, and it has been considered as the maximum amount of resources that the cell can devolve to ribosome synthesis (Scott et al., 2010). For P5, the value of the intercept decreases at increasing nutrient content. This could point to the fact that in richer media the competition for resources is stronger because there are more kinds of proteins being synthesized, therefore decreasing the resources available for P5 transcription and translation, while in the poor media only the strong promoters are able to drive protein synthesis. This should be verified with the study of different promoters in different growth media.

Until now we observed a constitutive promoter behaving under translation inhibition like a ribosomal one, and we confirmed our observation with several tests. It remains to be determined whether there are detectable differences in the induced response on promoters located in different parts of the chromosome. We measured therefore the GFP concentration for the P5 promoter at different chromosomal positions in one growth rate and at different chloramphenicol concentrations. The result is shown in Figure 27.

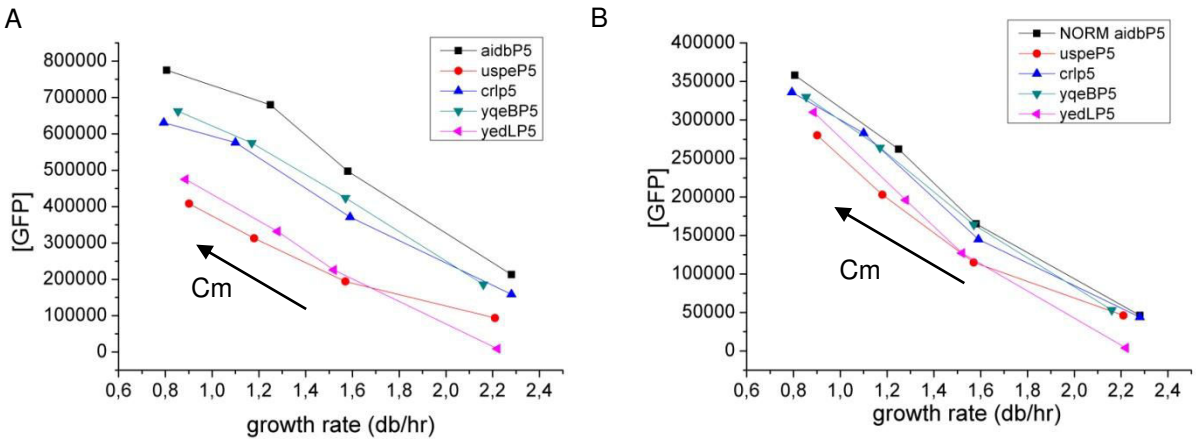


Figure 27 Protein concentration as a function of growth rate for the P5 strains growing in M9+glu04caa05 with different chloramphenicol concentrations before (A) and after normalization (B) by the gene copy number. A difference in the expression of uspe and yedL strains with respect to the others remains after correcting for the gene dosage.

For all the chromosomal positions there is an increase of GFP concentration at increasing translational inhibition. After normalization for the gene copy number, the GFP produced by the insertions close to the terminus (yedL and uspE) appears lower than the fluorescence resulting from the other strains. It could be a sign of the displacement of the available RNAP pool (RNAPfree) and ribosomes toward the

origin, where ribosomal promoters are located (Berger et al., 2010). However, a more accurate measurement of gene copy number by rtPCR is required in order to confirm possible effects of chloramphenicol. It is anyway interesting to test whether this effect of chromosomal position on gene expression detected for P5 exists also for P1 insertions, or whether the expression of this ribosomal promoter remains unaffected by the location on the chromosome. We are currently trying to answer this question.

6.2.4 Effect of a nutritional upshift on P1 and P5 expression as a function of chromosomal position

As I described in the introduction, bacteria adapt to abrupt changes in growth conditions by modifying the protein composition, the amount of ribosomes and RNAP, and the level of supercoiling. We can use our P1 strains in order to test whether all the parts of the chromosome react to the same extent and at the same time to a nutritional upshift, using the P5 strains as a control. The change in activity of the P5 promoter upon upshift can be regarded as a monitor of the level of available RNAP and the change in gene copy number, while P1 promoter expression is influenced also by the supercoiling level and by the concentration of ppGpp. For this reason, a change in P1 expression that is different from one chromosomal position to another could be ascribed to a different change in the supercoiling level of that region. To test at first the difference between P1 and P5, we grew the aidbP1 and aidbP5 cultures in minimal media supplemented with 0.2% casamino acids and then we transferred them to LB. Samples were harvested and analyzed by flow cytometry. The result is shown in Figure 28.

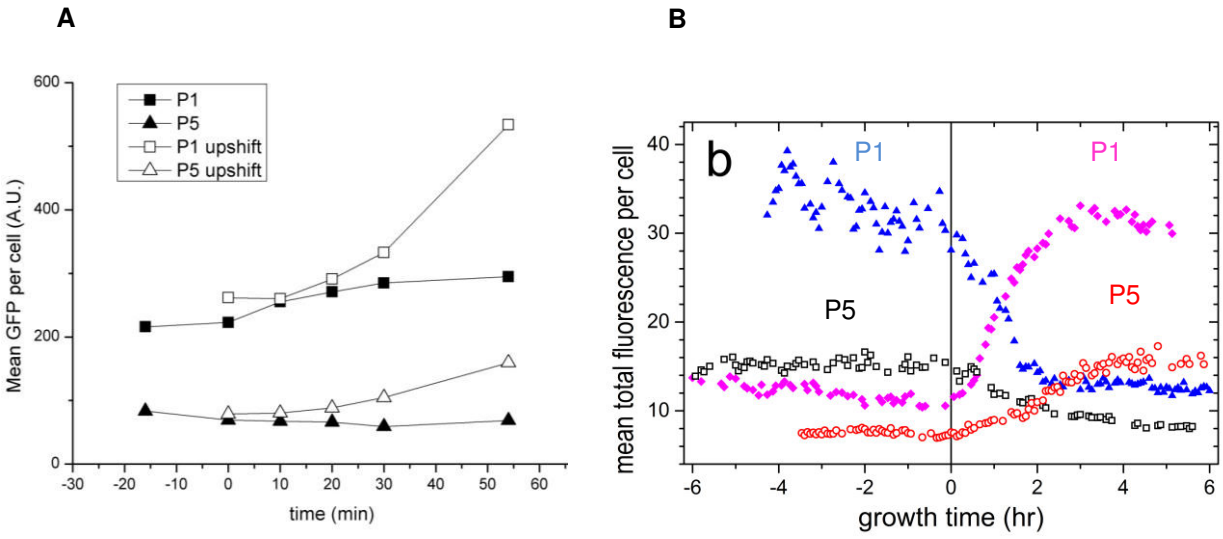


Figure 28 A) flow cytometry experiment and B) microfluidic experiment of changes in gene expression upon a change in growth condition. The plots show the amount of GFP per cell for P1 and P5 in the aidb strains upon nutritional upshift, plot A and magenta and red in plot B, and a downshift, blue and black in plot B. Upon upshift, both P1 and P5 expression increases, to a larger extent for P1. To be noticed the increased time resolution and the longer duration of the measurements one can obtain with a microfluidic device with respect to flow cytometry.

The mean GFP per cell increases after the shift to the richer medium for both the P1 and P5 strains. However, for P1, expression changes faster than P5, reflecting probably the combined effect of a dilution of ppGpp and an increase in supercoiling leading to an increase in P1 activity as a consequence of the upshift. The observed difference in P1 and P5 activity upon a nutritional upshift has been confirmed by microfluidic experiments with an increased time resolution (Long et al., 2014). The microfluidic device has clear advantages with respect to bulk experiment (more stable conditions, short time scales, rapidity in the media switch and single cell resolution) and would be the best choice for this kind of experiments, but plate reader and flow cytometer experiments provide necessary controls to ensure that growth in the microfluidic device is compatible with growth in standard conditions. In order to proceed with our study and to test whether there are differences in promoter induction for different chromosomal positions, we measured the expression of the P1 promoter for three different positions (aidb, uspe, yedl) for a shift from minimal media supplemented with casamino acids 0,2% (M9+CAA02) to richer media (M9+CAA05 or M9+GLU04CAA05), and followed the evolution of the samples using both the plate reader and the flow cytometer. Results are shown in Figure 29 and 30.

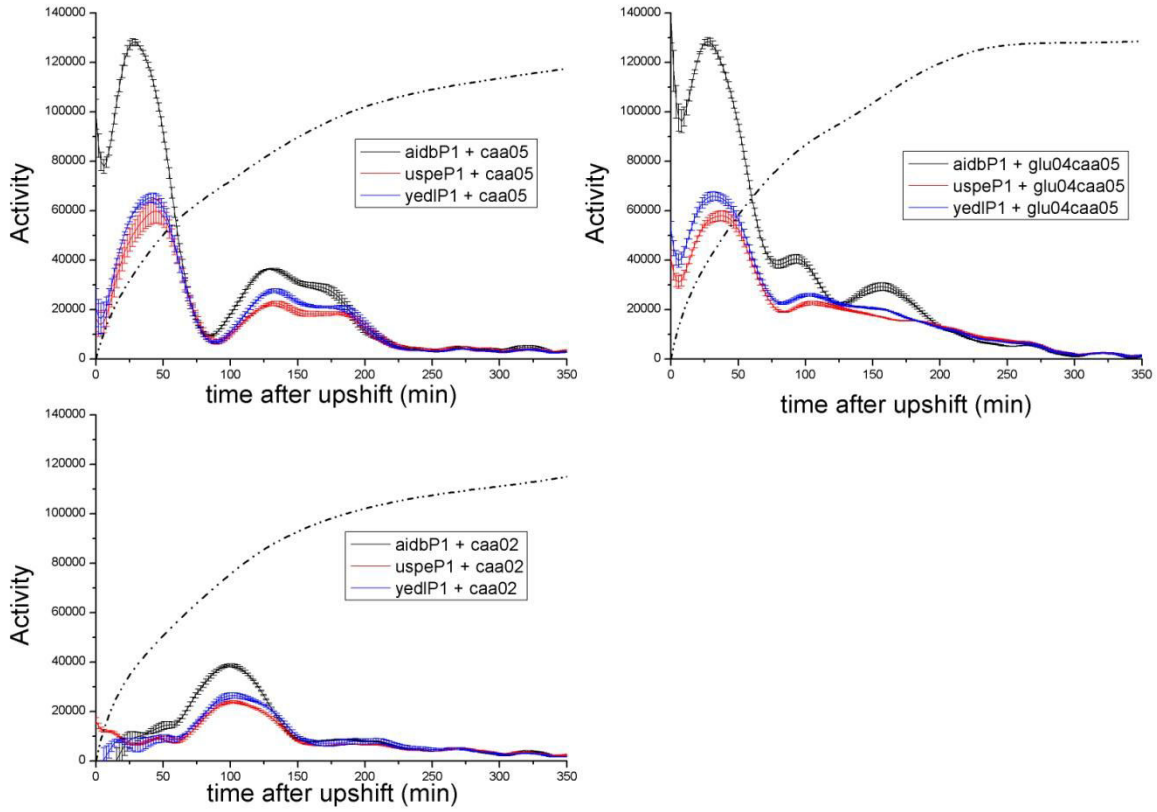


Figure 29 Plate reader measurements of the effect of a nutritional upshift on P1 promoter in aidb, yedL and uspe positions. Cells were grown until exponential phase in M9+CAA02 and then shifted to richer media (M9+CAA05 and M9+GLU04CAA05). The extent of induction is higher for the aidb position than for the others, but it could be due to an effect of gene copy number.

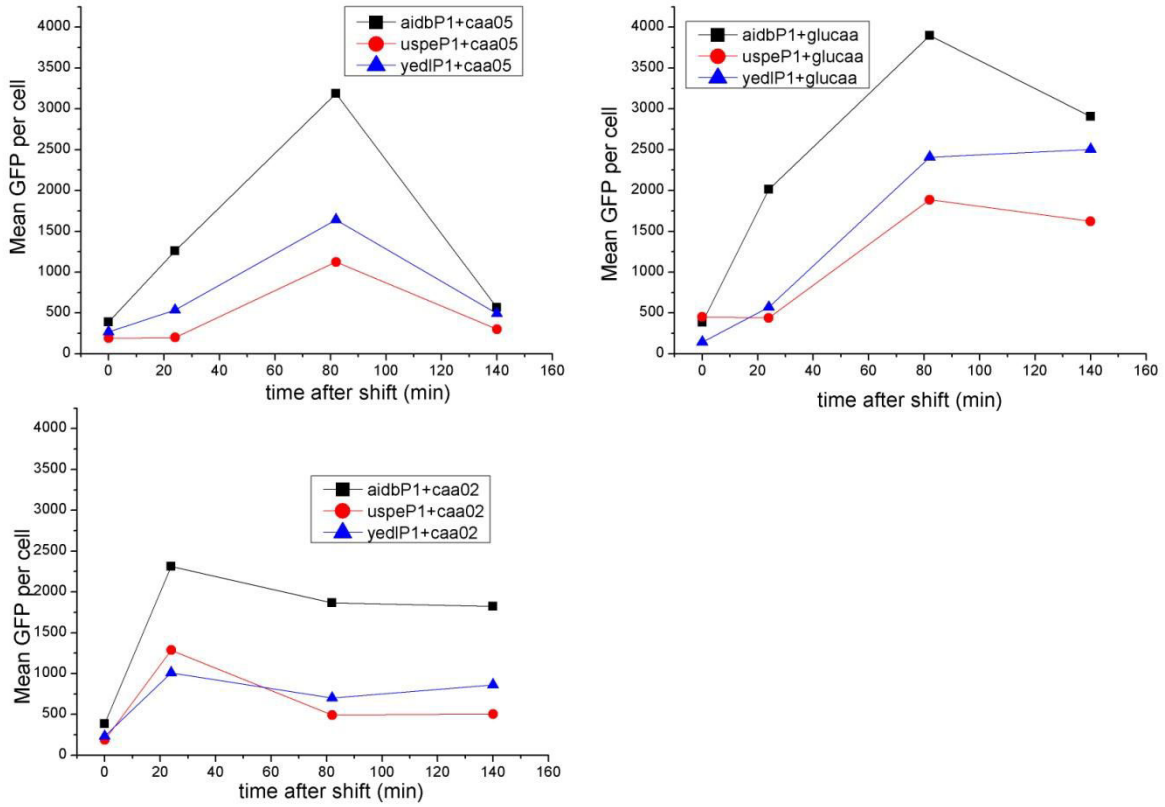


Figure 30 Flow cytometer measurements of the effect of a nutritional upshift on P1 promoter in aidb, yedL and uspe positions. Cells were grown until exponential phase in M9+CAA02 and then shifted to richer media (M9+CAA05 and M9+GLU04CAA05). Samples were harvested at different time points and the mean GFP per cell was measured. The extent of induction is higher for aidb position than for the others, but it could be due to an effect of gene copy number.

It appears clearly that the extent of induction upon nutritional upshift is higher for the insertion near the origin than for the insertion near the terminus. The rapidity of the increase in activity does not exclude the possibility that this effect is due only to a change in gene copy number, since it takes 20 minutes for DNA replication to shift from the old to the new rate (Kjeldgaard et al., 1958). The time required for adaptation to the new growth rate appears similar for the three P1 positions, and it is longer for the shift to the richer medium (M9+GLU04CAA05).

The same experiment should be repeated with the P5 strains, using P5 as a monitor of the change in gene copy number, in addition it should be realized in the microfluidic chamber, in order to have a fast monitoring of the change in fluorescence.

6.2.5 The influence of a neighboring gene on gene expression depends on the chromosomal position

In topologically constrained domains (Hardy and Cozzarelli, 2005), ongoing transcription generates increased negative supercoiling upstream of the promoter, and positive supercoiling downstream (Hanafi and Bossi, 2000; Liu and Wang, 1987), as shown in Figure 31A. In this situation, gyrase is required in order to release positive supercoiling and to allow transcription to proceed (Drlica, 1992).

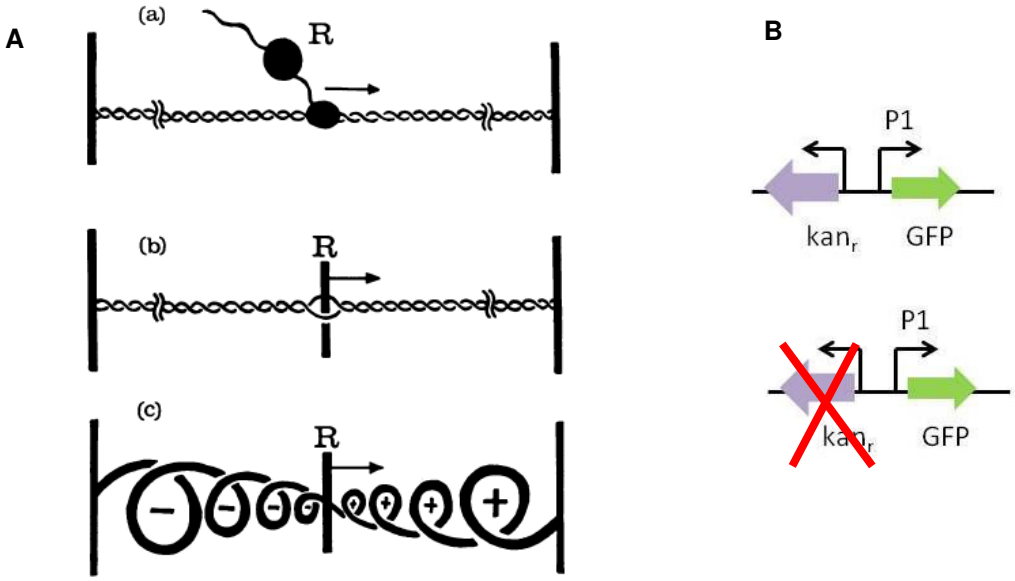


Figure 31 A) The graphical representation of the effect of ongoing transcription on the supercoiling of the DNA upstream and downstream the RNAP molecule. Positive supercoils are accumulated downstream the RNAP, and negative upstream (Liu and Wang, 1987). B) In order to measure the impact of transcription in different chromosomal positions, in our analysis we compare the expression of the reporter gene with or without the kanamycin divergent gene.

In addition, transcription from a neighbor gene can influence the expression of a downstream gene by competition for RNAP, transcriptional interference (Callen et al., 2004; Sneppen et al., 2005). On this basis, we asked whether the presence of the kanamycin resistance gene cassette (kanR) could affect the expression of the P1 and P5 promoters, and whether this influence could depend on chromosomal position. We therefore removed the kanR from our strains using the FLP recombinase protocol (Datsenko and Wanner, 2000), and we compared the expression of the promoter as a function of time with and without the resistance gene. For P1, we expected to detect a decrease in promoter activity once kanR has been removed, due to a decrease of induced negative supercoiling in the P1 promoter region. At the same time, the absence of a diverging gene could increase the activity of the other

promoter because of reduced competition for RNAP. We measured therefore the activity of the P1 promoter in different chromosomal positions in presence or in absence of the kanR, and compared these results with the ones obtained for P5. We expect P5 not to respond in the same way as P1, since it is not as sensitive to changes in negative supercoiling. Figure 32 shows the outcome of this experiment for the P1 promoter and Figure 33 the result for the P5 promoter.

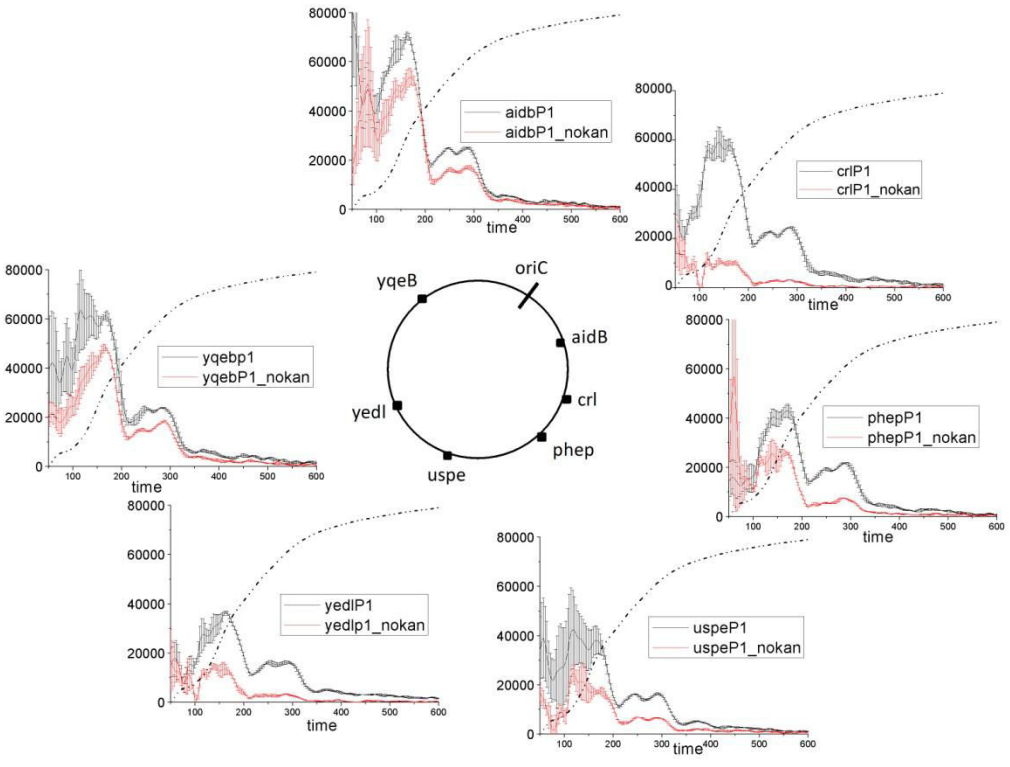


Figure 32 P1 promoter activity in different chromosomal positions in the presence (black) or in absence (red) of the divergent kanamycin resistance gene. Strains were growing in M9+CAA02 at 37°C in the plate reader. P1 activity is increased by the presence of kanR to a different extent as a function of chromosomal position.

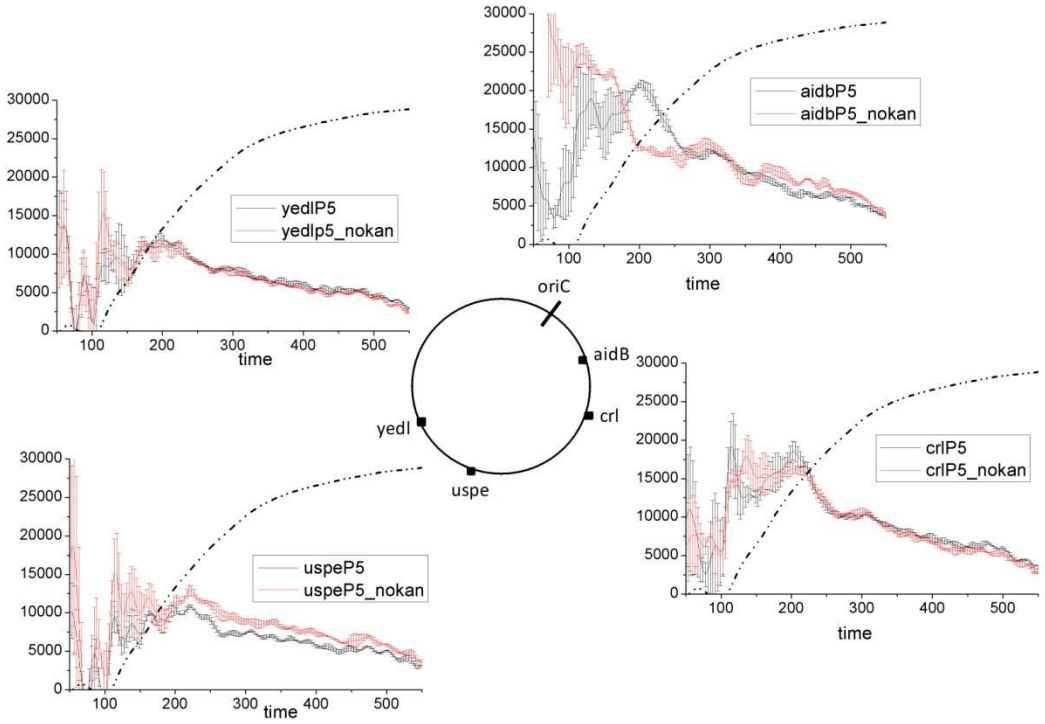


Figure 33 P5 promoter activity in different chromosomal positions in the presence (black) or in absence (red) of the divergent kanR. Strains were growing in M9+CAA02 at 37°C in the plate reader. P5 activity remains unaffected or slightly increases in absence of the kanamycin gene.

The activity of the P1 promoter is enhanced by the presence of the kanamycin resistance gene, being the activity 20% and even 80% higher than the correspondent strain where kanR has been deleted (see Figure 34). For P5 there is not a strong change in promoter activity with or without the divergent resistance gene but in the uspE position the activity in presence of the resistance gene is 30% lower than the promoter activity in its absence.

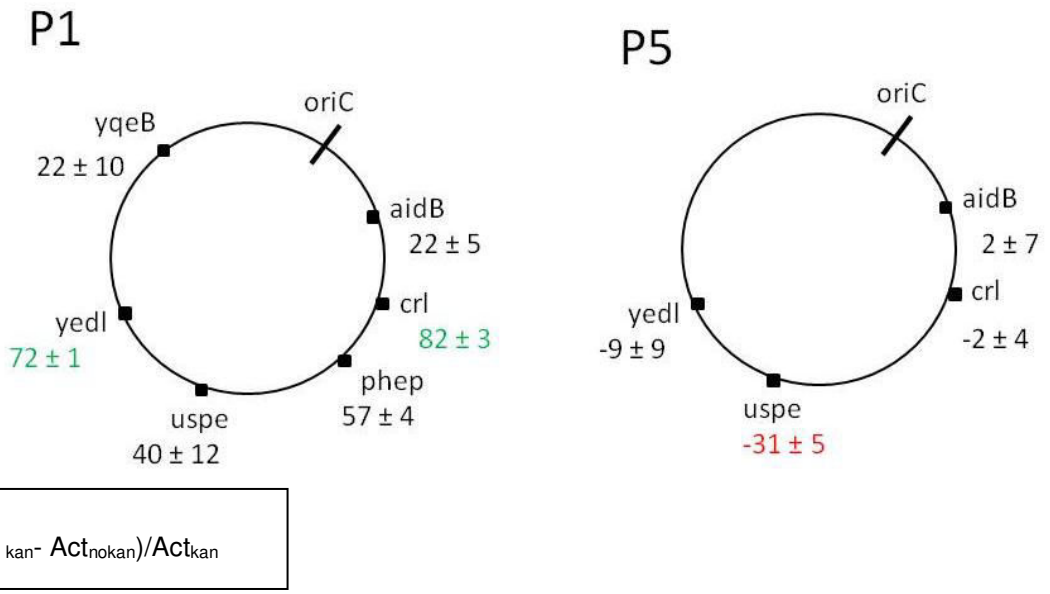


Figure 34 The difference in percentage between the integrals of the area below the promoter activity curve in presence or in absence of the kanR gene for two independent experiments. P1 activity is enhanced by the presence of kanR, while the effect of the neighbor gene on P5 expression is either none or negative.

The absence of the kanR gene has the opposite effects on P1 and P5, decreasing activity for the former, and leaving it unchanged or even increasing it in the latter. The decrease in P1 activity consequent to the absence of the resistance gene follows the behavior that is expected as a consequence of the decreased induced negative supercoiling, and the independence of P5 with respect to supercoiling can be the reason of the difference in the change between these two promoters. From the data shown in Figure 34, it is possible to notice that the change in P1 activity is weaker near the origin of replication. This could be the result of a higher level of supercoiling or a higher amount of RNAP near the origin, buffering the effect of increased supercoiling in the presence of the kanR gene.

A reduced concentration of RNAP polymerase near the terminus could explain the result found for uspeP5: in this case, the lack of kanamycin is increasing P5 expression because the competition for RNAP is released.

The reason for which P1 expression in yedL and in crl is so reduced in absence of kanR is not evident. yedL is located in the ter macrodomain between two convergent genes of unknown function , while crl is located inside the non-structured right domain, between the *phoE* gene, coding for a porin, and the *crl* gene, which stimulates the binding of the σ^{70} and σ^{s} factors to core RNAP (Gaal et al., 2006). If

there were an influence of transcriptionally silent high protein occupancy domains (tsEPODs) on these two insertions, the presence of active transcription of the kanamycin gene could contribute to weaken local protein binding, release the repression and increase P1 expression. However a similar effect should also be observed for P5 at these positions. Table 5 lists the chromosomal insertions and the genes flanking them. In the work by Bryant et al. it was found that high promoter activity in some specific positions on the chromosome was a consequence of the action of DNA gyrase (Bryant et al., 2014), this could be the case also for the crl and yedL insertions. The study of more chromosomal insertions and the use of drugs changing the activity of topoisomerases and thus the level of supercoiling could help in elucidating the mechanism causing the detected difference in gene expression in these two positions.

Insertion	**Orientation of promoter-GFP**	**Gene1**	**Insertion position**	**Gene2**
aidB	-1	aidB	4413923	yifN
crl	-1	crl	258230	phoE
phep	-1	pheP	602558	ybdG
yedL	-1	yedL	2009103	yedN_2
yqeB	-1	yqeA	3010636	yqeB
uspE	-1	ynaJ	1395696	uspe

Table 5 List of the chromosomal insertions of the complex kan+promoter+GFP and of the genes at the two sides of the insertion.

7 Conclusions and future perspectives

The goal of this work was to study the organization of chromosomal structure by analyzing the expression of a fluorescent reporter gene associated with different promoters as a function of chromosomal positions. By studying the expression driven by *Phns* as a function of chromosomal position in different growth conditions I was able to put in evidence the fact that the extent of the repression carried out by a global silencer such as H-NS depends both on the position of the regulated gene and on the growth conditions of the cell. For a highly abundant protein such that H-NS the repression of a target does not appear to depend directly on the spatial proximity of the target to the regulator's gene. The strength of the repression was mediated via the local properties of the chromosomal position, namely the higher than average AT content, and by the physiological state of the cell. At slow growth, or at entry into stationary phase, the detected repression by H-NS increased and extended also to a region close to the origin of replication, always in a way modulated by a local sequence effect. This could be a confirmation of the gradient model proposed by Sobetzko et al, where, according to the state of the cell, the level of negative chromosomal supercoiling is more or less high, and therefore the influence of H-NS can't (at high supercoiling states) or can (when the energy in the cell is lowered) influence the entire chromosome (Sobetzko et al., 2012).

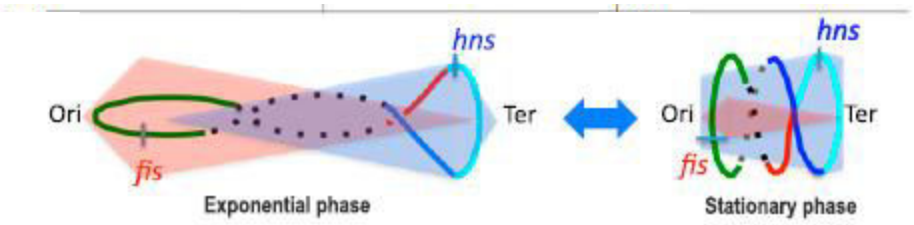


Figure 35 The gradient model from (Sobetzko et al., 2012). In exponential phase, the energy level and the negative supercoiling in the cell are high. In addition, H-NS has to compete with FIS for regulating target genes; therefore H-NS activity is mainly concentrated toward the terminus. At the entry into stationary phase, FIS concentration, cell energy and chromosomal supercoiling decrease, and H-NS extends its influence on the entire chromosome.

By studying P1 expression as a function of chromosomal position we wanted to measure the differences in supercoiling level in the different chromosomal locations. At equilibrium there is little difference in promoter activity between the different positions, even when looking as a function of

growth rate and growth phase. This result underlines the specificity of the effect that we have observed using the *Phns* reporter to the activity of the H-NS protein.

In order to detect possible change in the local level of supercoiling we decided to concentrate on experiments that cause a change in the level of supercoiling and thus in the transcription activity, such as the nutritional upshift experiments. The first results obtained with the upshift experiments have been presented in this thesis. It would also be interesting to modulate the dependence of P1 on ppGpp by mutating the discriminator region (Haugen et al., 2006), since the regulation of P1 by this nucleotide could mask the effect of changes in supercoiling. By removing the kanR cassette we were nevertheless able to reveal effects on P1 expression that depend on the chromosomal position that could be linked to local difference in the supercoiling level.

In order to have a negative control for P1 behavior we chose the strong, constitutive, T5 phage P5 promoter. Surprisingly, this promoter actually exceeded our expectations, becoming more interesting than a mere control. Actually we discovered that upon inhibition of translation the protein concentration driven by this promoter increased with increasing translational inhibition, to a similar extent than the one for the ribosomal P1 promoter. This was not expected, because in the literature it is commonly accepted that ribosomal promoter are induced during inhibition of translation in order to sustain growth even at reduced ribosome activity, but the amount of the other proteins should decrease due to the shift of transcriptional and translational resources toward ribosomes synthesis (Scott et al., 2010). This deviation from standard constitutive behavior is similar to what had been shown about another strong promoter, lacUV5 (Wanner et al., 1977), and about the constitutive ribosomal promoter P2 (Dennis et al., 2004). The concentration of protein synthesized by lacUV5 has been shown to be constant at changing growth rates, while one would expect that, being a constitutive promoter, its product should be diluted at faster growth (Klumpp et al., 2009). The activity of the P2 promoter even increases at increasing growth rates. In an effort to conciliate our result and the previous ones on lacUV5 and P2 with the model proposed by Klumpp et al., we can suggest that strong constitutive promoters can compete for the pool of free RNAP almost as well as ribosomal promoters, therefore their activity does not remain constant as a function of growth rate and their product is not diluted more at fast growth when the amount of free RNAP increases (HANS BREMER, 1996; Klumpp and Hwa, 2008). We could therefore proceed in this direction, both via the study of other promoters and via mathematical

simulations, and try to give a new definition of constitutive promoter and a new idea of resources partitioning based on promoter strength.

Appendix

I. Materials and methods

A. P5-P1STRAINS CONSTRUCTION

The medium-low copy plasmid pkk-GFPmut2 (Bouffartigues et al., 2007; Saggioro et al., 2013) was used for construction of rrnbP1-GFP and P5-GFP fusion. rrnBP1 was amplified by PCR from genomic DNA, while P5 was amplified by PCR from the PGEM plasmid (gift of Marta de Frutos). The promoter sequence was inserted in the XmaI-XbaI digested pkk plasmid. The pkk plasmid was then digested by XmaI-HindIII (located the first upstream the promoter, and the second downstream the GFP gene), and transform in the XmaI-HindIII digested pdocK (Lee et al., 2009). Both steps were verified by sequencing. From pdocK, the fragment promoter+GFP+Kan was amplified by PCR with primers with 50bp ends homologous to the chromosomal region of interest. The purified PCR product was then transformed via electroporation in BW25113 cells prepared according to the protocol of Datsenko and Wanner (Datsenko and Wanner, 2000). All the insertions were targeted in between convergent genes, in order to limit the perturbation on the environment, as well as the influence of the environment on our construct. The insertions were verified by PCR and by sequencing. For some of them, kanamycin resistance gene was removed by using FLP-helper plasmid pCP20. Below I list the plasmid sequences, as well as the sequences of the primers used for chromosomal insertion.

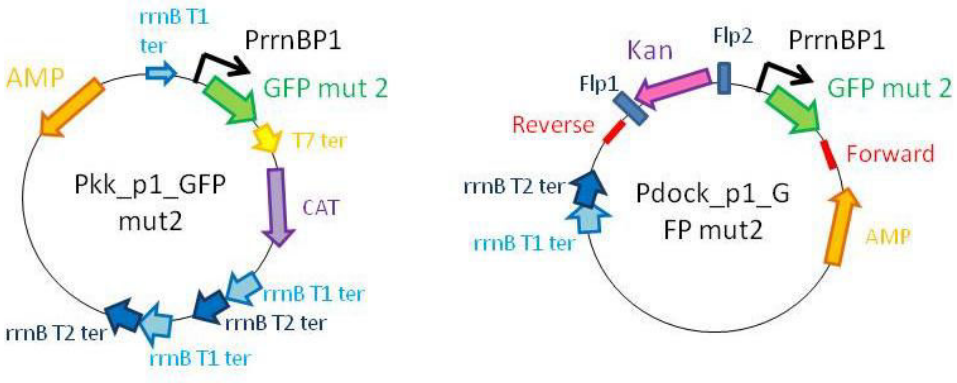


Figure A1 Schematic representation of pkk and pdoc-k plasmids. The resistance genes (AMP, Kan and CAT), the terminators (ter), the FLP sites (Flp1 and Flp2), the promoter and the GFP reporter gene and the annealing sites of the primers used to amplify the construct to be inserted in the chromosome (Reverse and Forward) are annotated.

B. PKK-RRNBP1-GFPMUT2 PLASMID

GTGCCACCTGACGTCTAAGAAACCATTATTATCATGACATTAACCTATAAAAATAGGCGTATCACGAGGCCCT
TTCGTCTTCAAGAATTCCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATC T1ter
TGTTGTTTGTCGGTGAACGCTCTCCTGAGTAGGACAAATCCGCCGGGAGCGGATTTGAACGTTGCGAAGCAA
CGGCCCGGAGGGTGGCGGGCAGGACGCCCGCCATAAACTGCCAGGGAATTCCCGGGTTGCGCGGTCAGA T2ter
AAATTATTTTAAATTTCCTCTTGTCAGGCCGGAATAACTCCCTATAATGCGCCACCACTGACATCTAGAAATAA PrrnBP1
TTTTGTTTAACTTTAAGAAGGAGATATACATATGAGTAAAGGAGAAGAACTTTTCACTGGAGTTGTCCCAATTC
TTGTTGAATTAGATGGTGATGTTAATGGGCACAAATTTTCTGTCAGTGGAGAGGGTGAAGGTGATGCAACATA
CGGAAAACTTACCCTTAAATTTATTTGCACTACTGGAAAACTACCTGTTCCATGGCCAACACTTGTCACTACTT
TCGCGTATGGTCTTCAATGCTTTGCGAGATACCCAGATCATATGAAACAGCATGACTTTTTCAAGAGTGCCAT GFP mut2
GCCCGAAGGTTATGTACAGGAAAGAACTATATTTTTCAAAGATGACGGGAACTACAAGACACGTGCTGAAGT
CAAGTTTGAAGGTGATACCCTTGTTAATAGAATCGAGTTAAAAGGTATTGATTTTAAAGAAGATGGAAACATTC
TTGGACACAAATTGGAATACAACTATAACTCACACAATGTATACATCATGGCAGACAAACAAAAGAATGGAAT
CAAAGTTAACTTCAAAATTAGACACAACATTGAAGATGGAAGCGTTCAACTAGCAGACCATTATCAACAAAATA
CTCCAATTGGCGATGGCCCTGTCCTTTTACCAGACAACCATTACCTGTCCACACAATCTGCCCTTTCGAAAGA
TCCCAACGAAAAGAGAGACCACATGGTCCTTCTTGAGTTTGTAACAGCTGCTGGGATTACACATGGCATGGA
TGAACTATACAAACACATGGCATGGATGAACTGTACAACTGAGGATCCGGCTGCTAACAAAGCCCGAAAGGA
AGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGA T7ter
GGGGTTTTTTGCTGAAAGGAGGAACTATATCCGGATATCCCGCAAGAGGCCCGGCAGTACCGGCATAACCA
AGCCTATGCCTACAGCATCCAGGGTGACGGTGCCGAGGATGACGATGAGCGCATTGTTAGATTTCATACACG
GTGCCTGACTGCGTTAGCAATTTAACTGTGATAAACTACCGCATTAAAGCTTGAGTAGGACAAATCCGCCGA
GCTTCGACGAGATTTTCAGGAGCTAAGGAAGCTAAAATGGAGAAAAAAATCACTGGATATACCACCGTTGATA
TATCCCAATCGCATCGTAAAGAACATTTTGAGGCATTTCAGTCAGTTGCTCAATGTACCTATAACCAGACCGT CAT
TCAGCTGGATATTACGGCCTTTTTAAAGACCGTAAAGAAAAATAAGCACAAGTTTTATCCGGCCTTTATTCACA
TTCTTGCCCGCCTGATGAATGCTCATCCGGAATTCCGTATGGCAATGAAAGACGGTGAGCTGGTGATATGGG

ATAGTGTTCACCCTTGTTACACCGTTTTCCATGAGCAAACTGAAACGTTTTCATCGCTCTGGAGTGAATACCA
CGACGATTTCCGGCAGTTTCTACACATATATTCGCAAGATGTGGCGTGTTACGGTGAAAACCTGGCCTATTTC
CCTAAAGGGTTTATTGAGAATATGTTTTTCGTCTCAGCCAATCCCTGGGTGAGTTTCACCAGTTTTGATTTAAA
CGTGGCCAATATGGACAACTTCTTCGCCCCCGTTTTCACCATGGGCAAATATTATACGCAAGGCGACAAGGT
GCTGATGCCGCTGGCGATTCAGGTTCATCATGCCGTCTGTGATGGCTTCCATGTCGGCAGAATGCTTAATGA
ATTACAACAGTACTGCGATGAGTGGCAGGGCGGGGCGTAATTTTTTTAAGGCAGTTATTGGTGCCCTTAAAC
GCCTGGTGCTACGCCTGAATAAGTGATAATAAGCGGATGAATGGCAGAAATTCGTCGAGGCGGCACCTCGC
TAACGGATTCACCACTCCAAGAATTGGAGCCAATCAATTCTTGCGGAGAACTGTGAATGCGCAAACCAACCC
TTGGCAGAACATATCCATCGCGTCCGCCATCTCCAGCAGCCGCACGCGGCGCATCTCGGCTGTTTTGGCGG
ATGAGAGAAGATTTTCAGCCTGATACAGATTAAATCAGAACGCAGAAGCGGTCTGATAAAACAGAATTTGCCT
GGCGGCAGTAGCGCGGTGGTCCCACCTGACCCCATGCCGAACTCAGAAGTGAAACGCCGTAGCGCCGATG
GTAGTGTGGGGTCTCCCCATGCGAGAGTAGGGAACTGCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAA
AGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCCTGAGTAGGACAAATCCGCCGGGAGC
GGATTTGAACGTTGCGAAGCAACGGCCCGGAGGGTGGCGGGCAGGACGCCCGCCATAAACTGCCAGGCAT
CAAATTAAGCAGAAGGCCATCCTGACGGATGGCCTTTTTGCGTTTCTACAAACTCTTCCTGTCGTCATATCTA
CAAGCCATCCCCCCACAGATACGGTAAACTAGCCTCGTTTTTGCATCAGGAAAGCAGCTGTTTTGGCGGATG
AGAGAAGATTTTCAGCCTGATACAGATTAAATCAGAACGCAGAAGCGGTCTGATAAAACAGAATTTGCCTGGC
GGCAGTAGCGCGGTGGTCCCACCTGACCCCATGCCGAACTCAGAAGTGAAACGCCGTAGCGCCGATGGTA
GTGTGGGGTCTCCCCATGCGAGAGTAGGGAACTGCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGA
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TTTGAACGTTGCGAAGCAACGGCCCGGAGGGTGGCGGGCAGGACGCCCGCCATAAACTGCCAGGCATCAA
ATTAAGCAGAAGGCCATCCTGACGGATGGCCTTTTTGCGTTTCTACAAACTCTTCCTGTCGTCATATCTACAA
GCCATCCCCCCACAGATACGGTAAACTAGCCTCGTTTTTGCATCAGGAAAGCAGTCGGGCAGCGTTGGGTC
CTGGCCACGGGTGCGCATGATCGTGCTCCTGTCGTTGAGGACCCGGCTAGGCTGGCGGGGTTGCCTTACT
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AGCAACAACATGAATGGTCTTCGGTTTCCGTGTTTCGTAAAGTCTGGAAACGCGGAAGTCAGCGCCCTGCAC
CATTATGTTCCGGATCTGCATCGCAGGATGCTGCTGGCTACCCTGTGGAACACCTACATCTGTATTAACGAA
GCGCTGGCATTGACCCTGAGTGATTTTTCTCTGGTCCCGCCGCATCCATACCGCCAGTTGTTTACCCTCACA
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GCAGACATCTGTGAATCGCTTCACGACCACGCTGATGAGCTTTACCGCAGCTGCCTCGCGCGTTTCGGTGAT
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CAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTTGGCGGGTGTCGGGGCGCAGCCATGACCCAGTCACGT
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GGTGTGAAATACCGCACAGATGCGTAAGGAGAAAATACCGCATCAGGCGCTCTTCCGCTTCCTCGCTCACTG
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ACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAA
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AGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCT
CCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCAA
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CGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATC
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TCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCA
AGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTC
AGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTT
AAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAAT
CAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATA
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TCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCG
CCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGT
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ACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGT
TGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATG
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CGAGTTGCTCTTGCCCGGCGTCAACACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCA
TTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCA
CTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCA
AAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATT
GAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGG
GTTCCGCGCACATTTCCCCGAAAA

Amp

C. PDOC-K-RRNBP1-GFPMUT2 PLASMID

GACGAAAGGGCCTCGTGATACGCCTATTTTTATAGGTTAATGTCATGATAATAATGGTTTCTTAGACGTCAGG
TGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGC
TCATGAGACAATAACCCTGATAAATGCTTCAATAATATTGAAAAAGGAAGAGTATGAGTATTCAACATTTCCGT
GTCGCCCTTATTCCCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAA
AAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTACATCGAACTGGATCTCAACAGCGGTAAGATCCTTG
AGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTATTATC
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Amp

Forward

GFP mut2

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CGTCTCCGGGAGCTGCATGTGTCAGAGGTTTTCACCGTCATCACCGAAACGCGCGA

D. PRIMERS FOR CHROMOSOMAL INSERTIONS

	Sequence
aidbF	GAATGATTTATTGCTGCGGGCGACGGGGGGAGTGTGTGTGtaaGCGTATACCAACTCAGCTTCCTTTCG
aidbR	CAATTTTCACATATTTCATTTAGTTAATCGAAACCAGCGTCGCATCAGTCGATGAGCTCGAGATATGAATATC
yqebF	CGTTTGTCATCAGTCTCAGGCCGGGTGGGTAATACCATCCGGCCATTTTCCCAACTCAGCTTCCTTTCG
yqebR	GCATCAGGGCGTGAAAGCGACAAAAGAAGTGCTGGAAGTGGCTtaaATATGAGCTCGAGATATGAATATC
crlF	GCGGATGATTTTCGTGACGAGCCGGTGAAGTTAACGGCGtgaGTGAACCAACTCAGCTTCCTTTCG
crlR	GACGTATCAGTTTtaaTGAATATTGCCGGATGTGATGCATCCGGCACATATGAGCTCGAGATATGAATATC
uspeF	GTAAGTAAAAAATAGGCCCGATAACTCGGGCCTTGTCAGTTATTGAACCAACTCAGCTTCCTTTCG
uspeR	GTATCAGACACCCGTTGAACTGGATGACGAAGAAGACGATtaaCGACTCATGAGCTCGAGATATGAATATC
yedlF	GCGAAAATAATGAATTGCCAtgaGCCAGACGCAGCACATTCTTGCATTCGACCAACTCAGCTTCCTTTCG
yedlR	CAAGGCGTTTCCTGCTGGTGATAAATAAAGGCGCAGCACGTCGAATGCATGAGCTCGAGATATGAATATC
phepF	ATTCCTGTTTATGGCATTTAAAACGCTGCGTCGGAAAtaaGGCATTCACGCCAACTCAGCTTCCTTTCG
phepR	GAATGGATGAGAGCGGTTTCGGATGGTTGACATCGTTTTGTCGGATGTAGATGAGCTCGAGATATGAATATC

Table A1 Sequences of the primers used for the chromosomal insertions. Each primer has the 3' part annealing to pdoc-k plasmid, and the 5' homologous to the region of interest in the chromosome.

E. PLATE READER ASSAY

Strains were grown overnight in LB supplemented with the appropriate antibiotic. In the morning, bacteria were diluted 1:10000 times in the appropriate medium with the automated liquid handler Beckman Coulter Biomek 3000. Samples were covered with mineral oil (Sigma) in order to avoid evaporation. The 96-well plate was then incubated in the plate reader Perkin Elmer Viktor 3, at 30°C or at 37°C, shaking. OD_{600} and fluorescence (485-535nm) were measured every 5 minutes for several hours. For the chloramphenicol experiments, in the morning the overnight cultures were diluted in the appropriate growth medium supplemented with chloramphenicol. Cultures were allowed to adapt for 6 hours in this growth conditions, then were diluted in the 96-well plate and measured in the plate reader. For the upshift experiment, cell grown in flask until exponential phase were pelleted, resuspended in the appropriate medium and loaded in the 96-well plate.

F. MATLAB ALGORITHM FOR PLATE READER DATA ANALYSIS

The core of the algorithm to analyse the 96-well plate experiment output is the WellReader implemented by Boyer et al. (Boyer et al., 2010). The data are loaded from the output file of the plate reader, and they are smoothed by using cubic smoothing splines. Background levels of absorbance and fluorescence from the wells with only the growth medium are subtracted to the other wells. To our purposes I customized the program, in order to align the OD curves such that the 15% of the OD values temporally coincide. We consequently shift the fluorescence curves as well, and we then subtract the autofluorescence signal from the BW25113 WT without any insertion. We then calculate the protein concentration, promoter activity and growth rate α as

$$[GFP] = \frac{Fluo}{OD600}$$

$$Activity = \frac{d(GFP)/dt}{OD600}$$

$$\alpha = \frac{d\,(OD600)/dt}{OD600}$$

G. FLOW CYTOMETER ASSAY

Overnight cultures were obtained by inoculating a single colony into M9 minimal medium supplemented with 0.2% casamino acids and Kanamycin 50 µg/ml, and by incubating them at 37°C shaking. For the upshift experiment, in the morning cultures were diluted 200-fold into fresh M9 minimal medium supplemented with 0.2% casamino acids, and grown to mid-log phase. Cultures were diluted 10 fold in the same medium, in order to extend the exponential phase. After 2 hours, each culture was split in two, cells were centrifuged 5' at 3500 rpm at room temperature, and then resuspended either in M9 minimal medium 0.2% casamino acids, or in LB. Samples were harvested every ten minutes, centrifuged for 5 minutes at 4°C at 3500 rpm, washed with PBS, fixed for 30' at room temperature with 2% Formaldehyde solution (Sigma) and washed three times with PBS. The fluorescence of the GFP/cell in the samples was then measured by flow cytometry. For the chloramphenicol experiment, the overnight culture was diluted 1:200 in the growth media supplemented with chloramphenicol. Cells were allowed to adapt until they reach exponential phase, then they were diluted again 1:10 in the growth medium with chloramphenicol, and grew until exponential phase. Samples were harvested and fixed as previously described. The samples were then measured on a FACS Calibur flow cytometer (BD Biosciences) with a 488 nm laser, using the 75 software BD CellQuest™ Pro. All instrument parameters were logarithmically amplified. Individual FSC, SSC and FL1 histograms were checked to be sure that the bell-shaped populations are not cut off on the display. An event rate of <1000 events per second was maintained in order to minimize the chance of coincidence and to improve population resolution. In the FSC versus SSC plot a live gate R1 was set around the bacterial population and a total of 20,000 events inside the gate were measured.

II. Noise as a measure of genome complexity?

The variability in the population of gene expression (noise) is connected with allocation of resources and survival. Is it more beneficial to spend energy and be prepared for a possible change in the environment or is it better to grow faster without allocating resources to alternative pathways? Is it more beneficial a tight control of gene expression in the population or is it more favorable to have a wide spectrum of expression? And, more connected with our analysis: can we derive useful information about chromosomal organization by studying the noise? Can the noise tell us if the position influences gene expression? Can the noise be a detector of RNAP distribution? I will describe first the theoretical findings about the noise, and then the questions to which the study of our reporters could give an answer.

A. WHY NOISE?

Gene expression affects the phenotype of the cell, and it is the result of a series of events during transcription and translation that require the formation of specific macromolecular complexes that can include for example RNA polymerase, transcription factors and ribosomes. Since the number of the molecules involved in these processes is usually small, gene expression is stochastic and leads to a variation in the amount of mRNAs and proteins per cell in a population of genetically identical organisms. This difference in gene expression from cell to cell is called “noise”. One of the first examples of this variation in a population is the “all-or-none” behavior of LacZ expression at low inducer concentrations, with some individual bacteria that are producing β–galactosidase at full rate and others that don’t produce the enzyme at all (Novick and Weiner, 1957). Another example is given by the differences in gene expression leading to a different lysis-lysogeny decision in lambda phage-infected *E. coli* cells (Arkin et al., 1998). Molecular-level fluctuations therefore can generate macroscopic differences in phenotype, and this variability can be an advantage for cell survival. In case of a irregularly fluctuating environment it has been shown that having different subsets of the total population with a metabolic pathway activated or inactivated is an advantage for the overall population (Kussell and Leibler, 2005). Another example of noise facilitating survival is the random switch of *E. coli* to a slow growth phenotype (persistence), which allows cells to survive to antibiotic exposure (Balaban et al., 2004). On the other hand, noise can be detrimental. It has been shown in mouse tissues that aging and noise in gene expression are correlated (Bahar et al., 2006), and in *C.*

elegans that the variation in the level of induction of a gene predicts the organism survival (Rea et al., 2005). Another hint that noise in gene expression is not always welcome is the existence of mechanisms devoted to minimize it, like in the robust chemotaxis network of *E. coli* (Kollmann et al., 2005) or in the plasmid copy number control (Paulsson and Ehrenberg, 2001).

Below, I will discuss the different sources of cell-to-cell variability in gene expression, and how it is possible to use noise to understand gene regulation.

B. DIFFERENT KINDS OF NOISE

Once the distribution of the number of proteins per cell is measured, the noise in the population is defined as

$$\eta = \frac{\sigma^2}{\mu^2}$$

with σ and μ the standard deviation and the mean of the distribution, respectively (see Figure A2).

In 2002 Elowitz et al. distinguished two different sources of noise in the cell (Elowitz et al., 2002). The first one, called extrinsic noise, is caused by the variations in the concentration and activity of molecules such as RNA polymerase and regulatory proteins. These factors are common for all the genes in a cell but differ from cell to cell. On the other hand, even with the same molecular components, promoters within the same cell would show differences in activity due to the stochasticity of the microscopic events leading to gene expression. This source of variation is called intrinsic noise. In order to discriminate between the two sources of variation, Elowitz et al inserted two copies of the same promoter in the *E. coli* genome, one driving the expression of the cyan fluorescent protein and the other the yellow fluorescent protein, and then measured with time-lapse microscopy the amount of each fluorescent molecule per cell. Extrinsic fluctuations are those affecting in the same way the two promoters, while intrinsic noise affects the two promoters independently. Extrinsic noise can be furthermore divided into global noise, that affect expression of all genes, and pathway-specific extrinsic noise, such as fluctuations in the abundance of a particular transcription factor that affects a specific gene but not another one (Raser and O'Shea, 2005). These different components of cell-to cell variability are summarized in Figure A2.

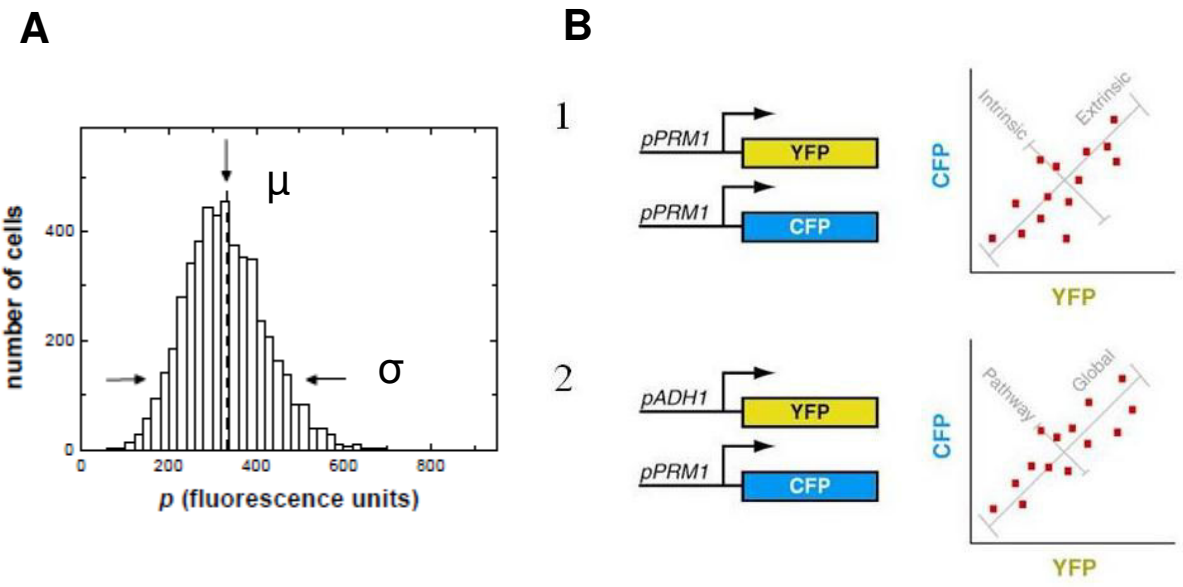

Figure A2 A) The distribution of fluorescence in a population, with μ the average value of the fluorescence per cell and σ the standard deviation of the distribution, adapted from (Ozbudak et al., 2002). B) Figure adapted from (Maheshri and O'Shea, 2007) showing the experimental setup for measurements of intrinsic and extrinsic noise. 1) Two different fluorescent genes are driven by the same promoter: the deviation of one with respect to the other is an effect of fluctuations at the promoter level (intrinsic noise), while the common variation is an effect of the physiological state of the cell (extrinsic noise). 2) By comparing the expression of two promoters involved in different cellular process, one can determine which component of noise is specific to the particular process, and therefore is changing the expression of one gene with respect to the other (pathway specific), and what is due to global fluctuation, affecting both promoters in the same way (global).

An overall inverse-proportional relationship between noise and gene expression is expected from uncorrelated stochastic systems. Elowitz et al. showed that strong constitutive promoters have low intrinsic noise, and also low overall cell-to-cell variation. When they lowered the rate of transcription, they detected an increase of both intrinsic and extrinsic noise. Raser and O'Shea applied the same method to the study of different promoters in *Saccharomyces cerevisiae* , and found that the total noise of gene expression was dominated by the contribution from extrinsic factors, and that noise intrinsic to gene expression was promoter-specific (Raser and O'Shea, 2004). The existence of a universal principle regulating noise was raised.

C. THERE IS A COMMON, GENERAL TREND OF NOISE, WITH SOME EXCEPTIONS

Different genome-wide studies have looked for a general behavior connecting noise and mean expression level of a gene. Newman et al. used high-throughput flow cytometry and a library of GFP-fusion proteins in *S. cerevisiae* to monitor protein levels and variance for cells growing in a rich growth

medium (Newman et al., 2006). In order to uncover the contribution of intrinsic and extrinsic noise they chose four promoters to be studied with two fluorophores. Intrinsic noise makes a significant contribution to the total variation of gene expression in the population. They also detected an inverse-proportional relationship between noise and protein abundance for low abundant proteins, while the distribution of abundant proteins was mainly governed by extrinsic noise as measured from the differences between cells (see Figure A3).

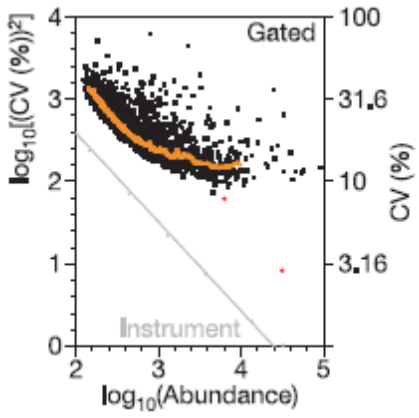

Figure A3 Figure from (Newman et al., 2006) describing the noise, defined as (σ/μ)2, as a function of protein abundance μ for a library of promoter in different growth media. For lowly expressed proteins, the noise shows a strong negative dependence on the protein abundance, while for highly expressed genes the noise reaches a plateau determined by extrinsic fluctuations.

Beyond this global behavior, they also found large differences in the coefficient of variation (CV=100* σ_p/μ_p) values for proteins expressed at similar levels. For instance, translation/ribosomal proteins and proteins whose production is regulated by transcription factors showed low noise, while other genes including enzymes that participate in ATP synthesis and those involved in stress-response were highly noisy. This suggests that transcriptional regulation plays an important role in determining noise level. The evidence of a proportional scaling of the noise with respect to the mean expression, with some deviations from this general behavior, was found in *S. cerevisiae* for cell facing different environments (Bar-Even et al., 2006). Further studies in yeast revealed a connection between the characteristics of a promoter and its level of intrinsic noise: highly noisy genes have a strong TATA-box and occupied proximal nucleosome (Field et al., 2008; Tirosh and Barkai, 2008), and the noise was decreased by mutating the TATA box, i.e. by reducing the burst size (see paragraph D) (Hornung et al., 2012; Raser and O'Shea, 2004).

In *E. coli*, a genome-wide analysis of noise based on a plasmid library showed that promoters can confer different levels of noise for the same level of expression (Silander et al., 2012). It was found that promoters associated with essential genes had a lower level of noise, while genes involved in energy metabolism of carbon sources and in adaptation to stress exhibited a higher level of noise. Taniguchi et al. analyzed the variation of protein and mRNA expression in a fluorescent protein fusion library with single-molecule precision (Taniguchi et al., 2010). They found, as a general behavior, that noise for low abundant proteins is mainly due to intrinsic noise and is inversely proportional to the mean protein number, while for highly expressed genes the noise was independent on the mean and flattened out to a plateau due to the extrinsic noise (see Figure A4). This result was similar to what was observed by Elowitz et al (see paragraph B).

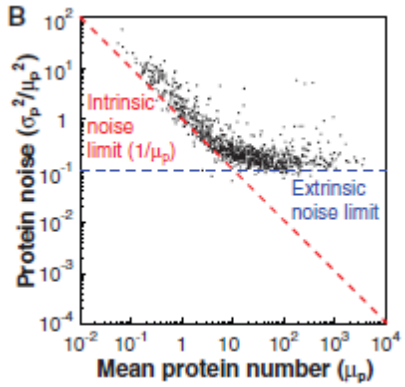

Figure A4 Figure from (Taniguchi et al., 2010) showing the linear scaling of noise in lowly expressed genes, whose expression is mainly governed by intrinsic noise, and the plateau of noise for highly expressed genes, whose variation of expression is due to the fluctuation of global cell condition.

In summary, two different kind of noise exist in the cell, one connected with the stochasticity of the events happening at the promoter level and leading to protein synthesis, and the other connected with the variation of global parameters such as free RNAP or ribosome concentration. The stronger influence of one component or on the other on the noise in gene expression depends on the level of gene expression itself: highly abundant proteins are distributed according to extrinsic noise, while the distribution of proteins present in low amount in the cell is governed by intrinsic noise. Beyond this global behavior, some differences in noise at the same protein expression were characterized for promoters involved in stress response (higher noise) or in essential genes (lower noise). However,

contrary to the clear effect detected in yeast, it is still not clear whether in *E. coli* the architecture of the promoter can influence the variation of gene expression in the population.

D. NOISE IN THE PROCESS OF PROTEIN SYNTHESIS

i. Noise at the transcription level

In a non-regulated (constitutive) gene, mRNA is produced at a constant rate K_r and destroyed with rate γ_r, with transcript production and degradation occurring in an uncorrelated fashion. If the number m of mRNA is large, the dynamics of the number of mRNA can be described by

$$dm/dt = K_r - \gamma_r * m$$

At the steady state, the mRNA molecules equilibrate, i.e. the probability of having m molecules of mRNA and producing another one is the same as having m+1 molecules and degrading one.

$$K_r * P(m) = \gamma_r * (m+1) * P(m+1)$$

that is possible if P(m) is a Poissonian distribution

$P(m) = e^{-\lambda}\frac{\lambda^m}{m!}$ with <m>=K_r/ γ_r

and the waiting time τ between two events is exponentially distributed (Thattai and van Oudenaarden, 2001). One would thus expect that a deviation from the Poisson behavior indicates that the gene is thus not constitutive, i.e. it is regulated. The time between two events in this case is then no longer exponentially distributed. The distribution is defined as 'bursty' if it has a higher frequency of short and long events compared to the poissonian distribution (the dotted line in Figure A5), while it is 'anti-bursty' (regular) if the frequency of interval of average length is higher than in the poissonian distribution.

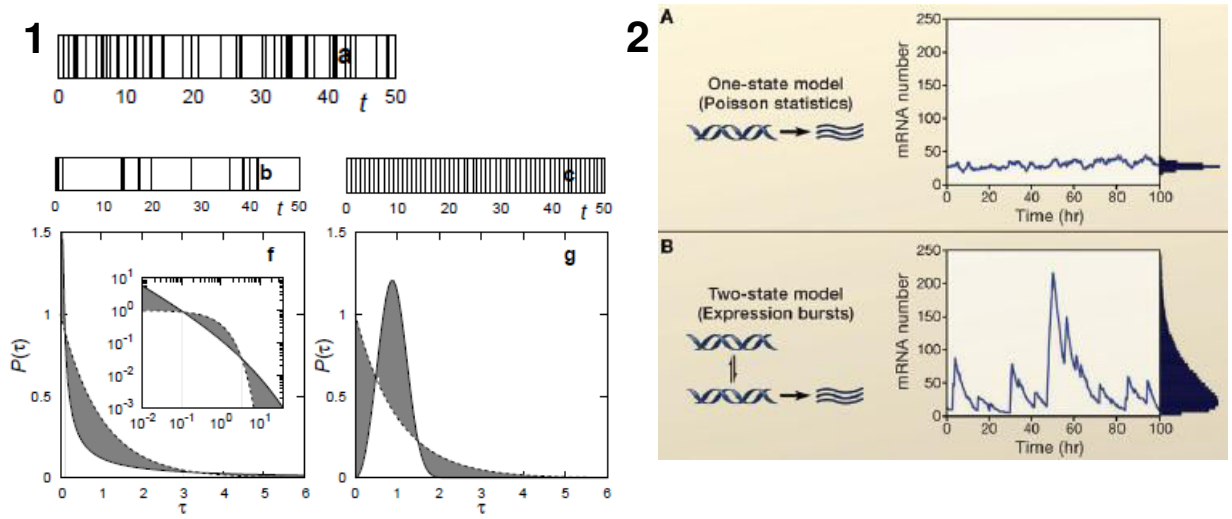

Figure A5 1) Figure adapted from (Goh and Barabàsi, 2006). Temporal pattern of events leading to a poissonian distribution (a) a bursty distribution (b) and an anti-bursty distribution (c). The temporal spacing between events in the three cases is shown below: a bursty distribution, in white in (f), is enriched in long and short interevent times and depleted in middle-length times with respect to a poissonian distribution (in gray). In (g), an anti-bursty distribution is enriched in middle-length interevent times with respect to a poissonian. 2) the difference in noise for a poissonian process and for a two-state bursty system, with the noise in the former being less than in the latter (Raj and van Oudenaarden, 2008).

Burstiness has been shown to exist so far in yeast (Zenklusen et al., 2008), mammalian cells (Ross et al., 1994) and in bacteria (Golding et al., 2005; Yu et al., 2006). The parameter that measures burstiness is the Fano factor, defined as the ratio between the variance and the mean of the distribution

$$b = \sigma^2/\mu$$

In case of a poissonian distribution, b=1. In *E. coli*, the mRNA distribution showed a higher Fano factor than a poissonian distribution (Taniguchi et al., 2010).

In the case of mRNA not produced with a constant probability per unit of time, the burstiness of the system can be explained by a two state model, in which the promoter switches stochastically between 'on' and 'off' states, where transcription can or cannot occur respectively (Raj et al., 2006; Raser and O'Shea, 2004). With a single copy of the gene, a random period of inactivity (OFF) is followed by a random period of activity (ON). If long OFF periods are followed by intense ON periods that produce a significant number of transcripts, transcription is said to occur in "bursts."(Golding et al., 2005)

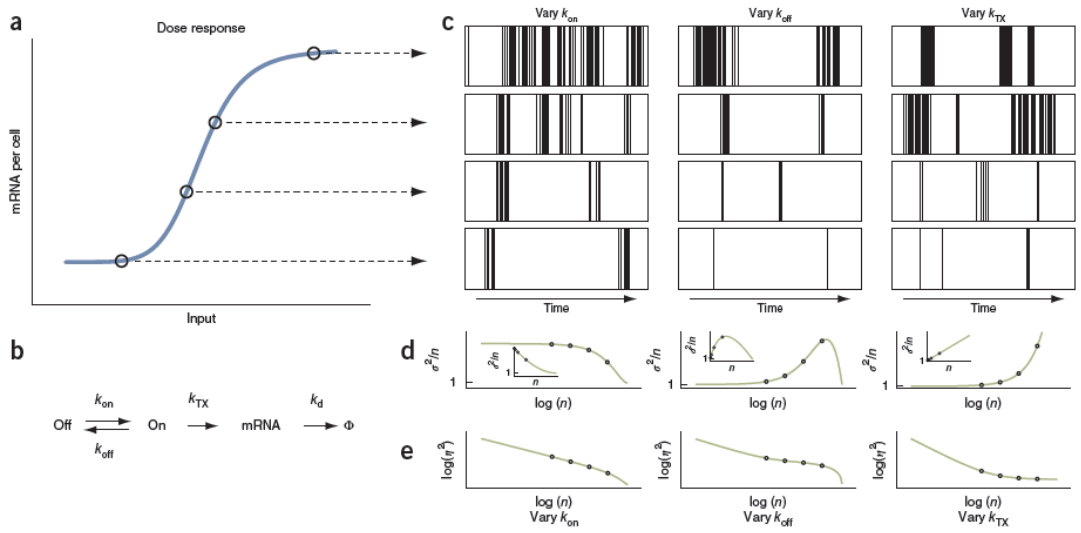

Figure A6 Figure from (So et al., 2011) illustrating how different series of expression events originated by a change in K_{on}, K_{off} or K_{tx} can result in the same value of mRNA per cell but are characterized by different Fano factor and noise.

The parameters of the two state model are K_{on}, the rate of promoter switching to the 'on' state, which determines the frequency of transcriptional burst; K_{off}, the rate of switching back to the 'off' state, which implies the duration of the bursts, and K_{tx}, the rate of mRNA production, which determines how many mRNA molecules are produced during the burst (So et al., 2011). The same mRNA level in the cell can be the result of different transcriptional time series (see Figure A6) and different modulations of the parameters involved (Munsky et al., 2012). These differences are quantified by the Fano factor. Increasing burst duration will increase mean expression without changing the noise, while increasing burst frequency will increase mean expression and decrease noise (Hornung et al., 2012).

So et al. measured the distribution of mRNA copy number at single-cell level for different promoters in different growth conditions, and calculated noise and Fano factor. Interestingly, they found a clear trend of noise and Fano factor as a function of the mean number of mRNA molecules that was the same for all the promoters and the growth conditions, in contrast with the promoter-dependence of noise that was shown in yeast (Hornung et al., 2012). This gene-independent behavior can be explained with a modulation of the K_{off} parameter, thus suggesting that the cell changes gene expression level by changing the duration of transcription bursts (see Figure A7). The observation that the noise in mRNA distribution at high mRNA number doesn't reach the plateau that would be expected in the presence of extrinsic noise, as it is in Figure A4, supports the idea that extrinsic noise

is more important at the protein level than at transcription level (Elowitz et al., 2002; Taniguchi et al., 2010).

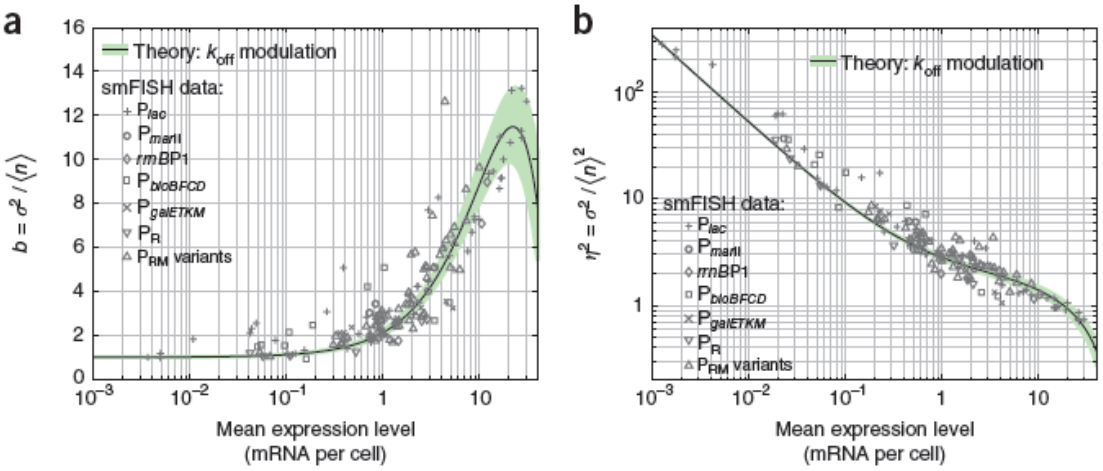

Figure A7 Figure from (So et al., 2011) illustrating the Fano factor (a) and the noise (b) for different promoters as a function of mRNA per cell. The trend of the Fano factor is compatible with the behavior predicted for the cell modulating the Koff in gene expression in order to vary the expression level.

ii. Noise at the translation level

Ozbudak et al. studied how in *B. Subtilis* the noise in gene expression was dependent on transcription and translation (Ozbudak et al., 2002). By using an inducible promoter driving *gfp* expression they were able to vary the transcriptional efficiency, while by mutating the ribosome binding site and initiation codon of *gfp* gene they were able to modulate the rate of translation. They found that high translation efficiency coupled with low transcription rate results in high noise, while low translation rates reduce fluctuations in protein concentration (see Figure A8). This result was compatible with a model in which proteins are produced in random bursts (Thattai and van Oudenaarden, 2001). They thus suggested that one of the mechanisms that the cell uses in order to lower the noise in gene expression is a low translation rate, like in the case of the important regulator *cya* (gene coding for cAMP).

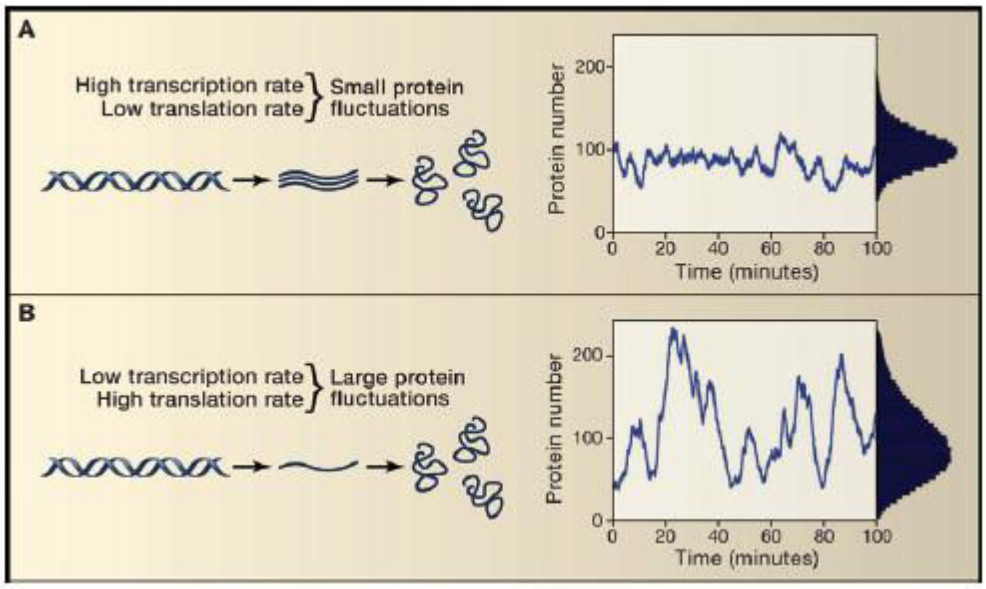

Figure A8 Figure from (Raj and van Oudenaarden, 2008) illustrating the amplitude of protein level variation in the population for a gene frequently transcribed but infrequently translated (A) and for a gene infrequently transcribed and frequently translated (B). In the second case the variation in the population is higher.

Cai et al. studied the real-time expression of β-Galactosidase in *E. coli* at single-cell level (Cai et al., 2006). They showed that protein production occurs in bursts, with the number of protein per burst following an exponential distribution. Since mRNA lifetime has been shown to be exponentially distributed (Kennell and Riezman, 1977), the exponential scaling of proteins per burst is seen as the effect of competition between translation and mRNA degradation. Swain et al. showed that in the case of efficient translation, i.e. number of proteins made per mRNA larger than 2, the intrinsic noise mainly depends on transcription (Swain et al., 2002). On the contrary, in the case of a less efficient translation the intrinsic noise is also dependent on translation. Therefore the coupling between transcription and translation rates influence the sensitivity of the gene expression to extrinsic and intrinsic noise, and therefore the variation of gene expression in the population.

iii. Noise propagation in networks

It is interesting to study whether the noise in a regulator can affect the noise in the target gene. By using multiple fluorescent reporters in synthetic genetic networks, it was shown that variability in the regulator was transmitted to the regulated gene (Pedraza and van Oudenaarden, 2005; Rosenfeld et al., 2005). In the case of a negative feedback loop, where the fluctuations in protein concentration are pushed back to the mean value, it was shown that the noise was reduced with respect to a constitutively expressed gene (Becskei and Serrano, 2000; Thattai and van Oudenaarden, 2001), as

shown in Figure A9. On the contrary, in the presence of positive feedback a small difference in the protein level can lead to a further activation of the gene, therefore increasing the variability in the population and eventually leading to bistable expression profiles (Acar et al., 2005; Becskei et al., 2001).

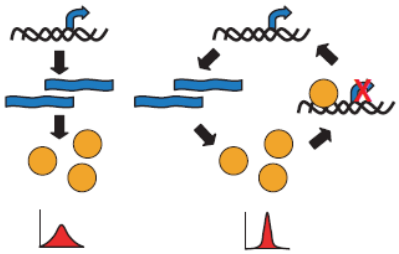

Figure A9 Figure from (Raser and O'Shea, 2005) describing the noise in protein level originated by a constitutive promoter, on the left, and by a gene negatively autoregulated. For the auto regulated promoter the variation in the population is lower.

With our reporters we can observe whether the chromosomal position has an impact on the variability of gene expression. The study of noise as a function of chromosomal position for a gene regulated by lac repressor didn't put in evidence any influence of the position on the noise (Block et al., 2012), but the promoters we are taking into account are different, therefore it is possible to have a different outcome. Moreover, by comparing P1 and P5 we can put in evidence whether the tight regulation on P1 expression lower the noise in gene expression with respect to a non-regulated promoter, and by analyzing the noise at different growth rates, i.e. with different gene copy number, we can detect whether the multiple copies of the gene are synchronized or not (Maheshri and O'Shea, 2007). We can also study if and to what extent the perturbation of supercoiling via nutritional upshift or via gyrase inhibition is transferred to the noise of P1 and P5 promoters.

E. EFFECTS OF POSITION ON THE NOISE: A FIRST ATTEMPT

As I described in the introduction, the complex structure of the chromosome could in principle affect the level of noise in gene expression due to a different accessibility of the promoter to transcription factors and RNA polymerase, due to the distance from a regulator, or due to neighbor genes effects (see chapter 1).

We measured the distribution of the GFP per cell in the population for P1 in three different positions (aidb, yedL and uspe) in exponential phase at different growth rates. Cultures were grown at 37°C shaking, and samples harvested in exponential phase were fixed with formaldehyde and analyzed by flow cytometry. The average GFP per cell μ and the width σ of the distribution were calculated. The results obtained for noise, defined as σ^2/μ^2, and for the Fano factor, defined as σ^2/μ, are shown in figure A10.

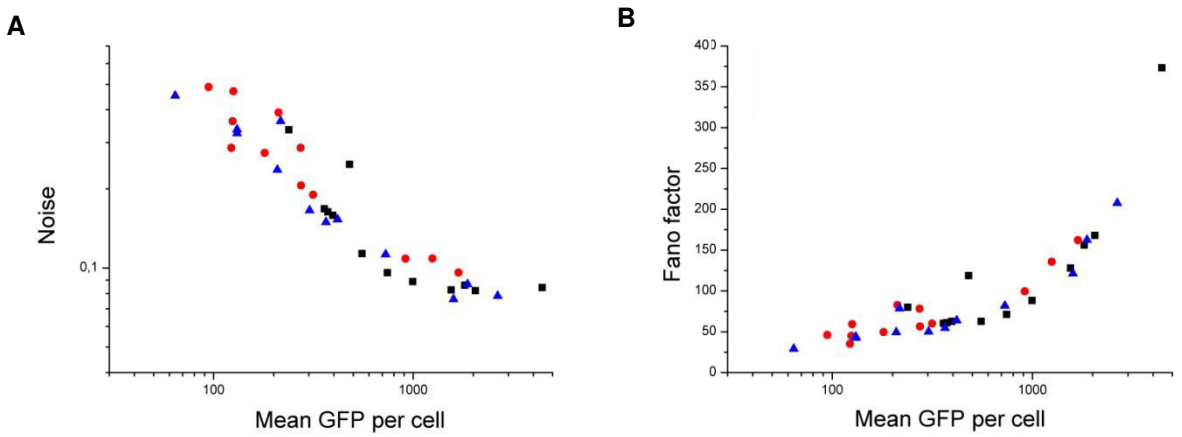

Figure A10 Noise (A) and Fano factor (B) for P1 promoter inserted in aidb (black), uspe (red) and yedL (blue) positions in exponential phase in different growth media. The noise do not appear to depend on chromosomal position, but it depends on the amount of GFP per cell. The Fano factor increases at increasing GFP levels following the trend correspondent to a change in the translation rate (So et al., 2011).

We can notice from the results that the noise follows the global trend that has been shown previously in the literature (Newman et al., 2006; Taniguchi et al., 2010), with noise decreasing at increasing promoter expression (intrinsic noise limit) and then reaching a plateau at high level of expression (extrinsic noise). The Fano factor can be calculated in order to decipher which parameter among K_{on}, K_{off} and K_t the cell is modulating in order to regulate the level of gene expression (So et al., 2011). From the comparison with the three different scenarios hypothesized in the So et al. paper, we can conclude that the cell changes GFP gene expression from the P1 promoter by changing the translation rate (see Figure A6). This is in agreement with the increase in the amount of ribosomes at fast growth (HANS BREMER, 1996), leading to higher translation rate. On the other hand, promoter activity for ribosomal promoters increases as well at fast growth (Klumpp and Hwa, 2008), involving a change in K_{on} and K_{off}. A comparison with P5 would be valuable in order to discriminate between modulations by the various parameters, and could also show whether the noise of this two differently regulated promoters at the same GFP level is different.

iv. Modulating noise for P1 and P5 promoters

K_{on} K_{tx}

$$R + P \rightleftarrows RP \rightarrow RPo \rightarrow RPel$$

K_{off}

Even if the detection of an influence of gene position on the noise in gene expression was not successful at the first attempt, we can think about out-of-equilibrium experiment and about mutations in the two promoters in order to create a system more sensitive. The formation of the RNAP-promoter complex at P1 is fast (Gourse, 1988), thanks to the presence of the UP element (Gourse et al., 2000), and unstable due to the presence of discriminator region, depending therefore on the level of ppGpp (Bartlett and Gourse, 1994; Zacharias et al., 1989) and of supercoiling (Chong et al., 2014). Once the RNAP is bound the transcription rate K_{tx} is fast (Jensen and Pedersen, 1990). For the P5 promoter on the other hand the binding of RNAP is fast, due to the UP element, and stable, due to the optimality of P5 promoter sequence (see paragraph 5.2 and 5.3). For the same reason, the transcription rate is slower than the one for P1. The translation rate for both P1 and P5 is fast due to the presence of a strong ribosome binding site (see Figure 22). A modulation of noise for the P1 promoter can be obtained by changing the level of ppGpp and of supercoiling, i.e.by affecting the K_{off} of P1 and the K_{tx} for P5 (see Figure 6), in addition by removing the UP element, and decreasing as a consequence the K_{on} of both promoters, or by adding the original FIS binding sites at P1 promoter, therefore stabilizing the complex RNAP-promoter, and finally by changing the sequence of the ribosome binding site, affecting in this way the translation rate. There is therefore a challenging and interesting path on the way to uncover the influence of gene position on gene expression.

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