Transcriptional regulation of nrdAB genes in E. coli – analysis of the new regulator NrdR

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Abstract

The enzyme ribonucleotide reductase (RNR) provides the building blocks necessary for DNA synthesis and repair. In *E. coli* there are three classes of RNRs. The oxygen dependent Ia reductase is encoded by the *nrdAB* gene, and its transcriptional regulation by NrdR is the target of this study. We experimentally demonstrated negative regulation of *nrdAB* expression by NrdR, using plasmids with *nrdAB* promoter *gfp* fusion in wild type and mutant (∆*nrdR*) strains. This repressor is a link between RNR inhibition with hydroxyurea and increased expression of *nrdAB* in *E. coli*.

We show that the expression of *nrdAB* promoter reveal a 3-fold lower *nrdAB* promoter activity in wild type than in mutant which shows the effect of NrdR repression. An increase by 2.4 fold in wt strain with plasmid containing all promoter elements (p34) treated with HU and 1.1 fold in mutant was observed. In addition, the presence of the intact plasmid (p38) in wt strain show 1.4 fold increase with addition of HU whereas in mutant only 1.1. The tight regulation of RNR and dNTP pools is monitored by Flow cytometry analysis where DNA/cell mass decrease was observed in wt and mutant strains with HU. The Flow cytometry also showed that DNA replication occurs faster in mutant strains than in the wild type.

Finally we show that inhibition of DNA-synthesis, by way of treatment with the chemical nalidixic acid, greatly stimulates expression of *nrdAB*, and strengthens the repressive efficacy of the NrdR.
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RNR-Ribonucleotide reductase

Every organism that synthesizes its own DNA, maintains a constant DNA/cell mass ratio through a complex regulatory mechanism. DNA synthesis is initiated at the origin of replication (a specific site in bacterial chromosome) and in *Escherichia coli* circular chromosome the replication precedes bidirectionally to elongate and duplicate the chromosome. The initiation and elongation processes are important parameters for the rate of DNA synthesis. The initiation step in *E. coli* depends on the protein DnaA and the elongation is dependent on the DNA polymerase III holoenzyme complex that incorporates dNTPs in the synthesized chain. In all organisms, the central protein that catalyses the conversion of nucleotides to deoxynucleotides is ribonucleotide reductase (RNR) (see review Herrick et al., 2007). Ribonucleotide reductase acts in the first reaction (the rate limiting step) of the *de novo* pathway for DNA synthesis by providing deoxyribonucleoside diphosphates (dNDPs). It contains a tyrosine-cysteine radical and uses thioredoxin and glutaredoxin to reduce ribonucleoside diphosphate, maintaining regulated balanced pool of DNA precursors during replication (Nelson and Cox, 2005). In the *Escherichia coli* genome, *nrdAB* (class Ia), *nrdHIEF* (class Ib) and *nrdDG* (class III) are the three operons encoding 3 different ribonucleotide reductases (RNRs) respectively. The major (class Ia oxygen dependent) reductase consists of two nonidentical subunits R1 (homodimers) and R2 encoded by *nrdA* and *nrdB* (Nelson and Cox, 2005, pg.870).
Regulation of RNR activity is achieved through allosteric control of the activity and specificity of RNR by nucleoside triphosphates effectors and by regulation at transcriptional level (see review Herrick et al., 2007).

Enzyme activity and substrate specificity of RNR is regulated at R1 subunit. R1 regulatory sites are primary regulation site and substrate specificity site. The primary regulation site binds either ATP, which activates the enzymes or dATP (product) which inactivates it. The second regulatory site binds effector molecules: ATP, dATP, dGTP and dTTP that regulate balanced pools of nucleotides.
The product acts as an effector molecule altering the specificity for a substrate by changing enzyme conformations. dTTP stimulates GDP reduction but inhibits UDP (dUTP is a precursor for dTTP). A high level of dGTP inhibits reduction of UDP (and following dTTP), GDP and CDP but stimulates ADP reduction. ATP stimulates reduction of CDP and UDP. dNTP pool imbalances result in replication abnormalities and mutations. (Wheeler et al., 2005)

The R2 subunit has binuclear iron (Fe$^{3+}$) cofactor which helps stabilize tyrosyl radicals. The importance of the tyrosine radical on R2 subunit is to generate an active site radical which stabilizes the cation formed after loss of water molecule in formation of dNDP from NDP. (*see figure 3*)

\[
\text{-} \text{O}^\bullet + \text{-XH} \rightleftharpoons \text{-} \text{OH} + \text{-X}^\bullet
\]

*Figure 2 Tyrosyl radical generates active site radical (-X$^\bullet$) which is involved in dNDP formation mechanism (Nelson and Cox, 2005, pg.870) In presence of HU (radical scavenger) the generation of active site free radical is inhibited which results in decreased dNDP formation.*
Figure 3 Proposed mechanism for RNR according to Lehninger 4th edition (Nelson and Cox, 2005 pg. 871).

An active-site radical formed by the reaction in figure 3 helps formation and stabilization of a cation radical. By oxidation of dithiol the radical cation is reduced generating active-site radical and forming deoxy product. The active site radical is then able to undergo another cycle. It is believed that the catalysis is initiated between Tyrosine 122 (in E.coli) in R1 and active site cysteine in R1. (Hogbom et al., 2003)
RNR is also feedback regulated at a transcriptional level. Its regulation is very complex and under study because any elevation of RNR expression as well as the dNTP pool contributes to abnormal cell behavior. This underlines the fact that the RNR is expressed when cell undergoes initiation of replication, DNA damage, nucleotide starvation and when RNR is damaged or inactivated by chemical agent or site mutations (see review Herrick., 2007).

The expression of \textit{nrdAB} operon is found to be regulated by binding of three regulatory proteins DnaA, Fis and ArgP to the specific sites on the promoter region under aerobic conditions. RNR is inhibited by hydroxyurea (HU), a free radical scavenger, which induces expression of \textit{nrdAB} and \textit{nrdHIEF} operons by an unknown mechanism. Moreover, hydroxyurea inhibits DNA polymerase at the replication fork by decreasing its substrate (dNTPs) concentration (Wheeler et al., 2004).

**Regulator proteins DnaA, Fis, and ArgP**

The \textit{nrdAB} operon is so far known to include four binding motifs, of which two primarily display activator roles; Fis, and ArgP and the regulatory role of DnaA has been argued. Detailed structural roles have not yet been defined for the two, but experiments have shown that they play a positive regulatory role in the expression of \textit{nrdAB}.

DnaA is essential for the initiation of chromosomal replication (oriC) in prokaryotes. DnaA binds to 9 bp consensus sequence \([TTA/TTNCACA]\) within \textit{oriC} named DnaA-binding box which results in opening the AT rich region of the double stranded DNA. In \textit{E. coli} DnaA binds ATP or ADP forming stable complex but only ATP-DnaA form is active in DNA strand opening during initiation and stabilizing the single stranded regions (see review Herrick et al., 2007).

Early studies on \textit{nrdAB} gene expression have shown that, DnaA has a positive effect on \textit{nrdAB} expression proving its role as an activator. By comparing wild-type and mutant with DnaA box-mutated \textit{nrdAB} promoter on single-copy plasmid, they observed lower expression of \textit{nrdAB} from the mutated promoter (see review Herrick et al., 2007).

The recent studies for the role of DnaA on \textit{nrdAB} expression have lead to a disagreement between different studies. According to Løbner-Olesen et al., inactivated DnaA mutant (\textit{dnaA46}) leads to depression of \textit{ndr}, whereas Gon et al., showed the opposite by using two DnaA mutant proteins inactivated.
One of the mutants had high ATPase activity and the other one has been defective in ATP binding. This study showed that high levels of ATP-DnaA form caused decrease in the transcription of \textit{nrdAB} and the low levels of ATP-DnaA, where the ADP-DnaA form is higher, the level of \textit{nrdAB} increases. In the same study it was also shown that ADP-DnaA has low amount of repression on \textit{nrdAB} (Gon et al., 2006). Augustin et al., demonstrated that DnaA is an activator by comparing wild type to DnaA box-mutated \textit{nrd} promoter fragments fused to \textit{lacZ} on single-copy plasmid (Augustin et al., 1994).

The \textit{nrd} promoter region contains two DnaA boxes and sequences conforming to ATP-DnaA boxes located right upstream of \textit{nrdAB}. DnaA binds to these boxes and regulates the transcription of the \textit{nrdAB} operon (Tuggle and Fuchs, 1986; Speck et al., 1999). During initiation mostly the DnaA is in ATP-DnaA bound form and represses the \textit{nrdAB} operon and on the other side down regulating the dNTPs pool. On the process of elongation the level of ATP-DnaA is decreased by the protein Hda which hydrolysis ATP-DnaA form to ADP-DnaA, by this the repression on \textit{nrdAB} decreases resulting in high levels of RNR required for the synthesis of DNA. ATP-DnaA always accumulates in the cell prior to the next cycle of replication resulting to continuously repression of \textit{nrdAB} transcription (Gon et al., 2006).

DnaA protein also regulates transcription of several other genes for example like the \textit{mioC} gene, which is located just next to \textit{oriC} and is negatively regulated (Asklund et al., 2004).

Presumably Fis (factor for inversion stimulation) enhances expression by bending of the DNA duplex, when bound to its respective upstream recognition sequences, thus lowering the dissociation constant of the RNA polymerase-promotor complex (Augustin et al., 1994). Fis is the most common nucleoid-associated protein during the logarithmic growth in rapidly growing \textit{E. coli} cells, but during the stationary phase it almost disappears and is low in slow growing cells. Its effect is widespread because it is able to interact with many DNA sites to alter DNA topology where many genes can be regulated either positively or negatively (Cohen, et al., 2005). Deletions in the \textit{nrd} promoter regulatory element where Fis binds showed five to six fold decrease in the expression of \textit{nrd-lac} fusion gene (Augustion et al., 1994).
The third transcriptional activator of \textit{nrdAB} is ArgP protein. Han et al., showed that overproduction of ArgP in vivo increases the expression of \textit{nrdAB} by four to five fold (Han et al., 1998).

Recently, a transcriptional regulator has been identified known as NrdR, which controls the expression of RNR genes by acting as a repressor. This has been concluded as a result experimental analysis by \textit{nrdR} gene mutations from \textit{E.coli} genome which resulted in elevated transcription of Ia and Ib genes (Torrents et al., 2007).

![Figure 4 Structure of \textit{E.coli} \textit{nrdAB} operon and promoter (preliminary sketch by Tove Atlung)](image)

**NrdR transcriptional repressor**

As mentioned above, NrdR transcriptional repressor binds to the tandem imperfect palindromic16 bp repeat sequences called NrdR boxes, conserved in eubacteria. NrdR binding motifs are located 5’ upstream region of all tree RNR genes and in the \textit{E. coli} there are two tandem NrdR boxes. Both repressor binding sites shows minor difference in their position of three RNR genes (\textit{figure 5}) that affects outcome of the expression. (Torrents et al., 2007)
Figure 5  *E.coli* (ECO) and *S. enterica* (STY) *nrdAB*, *nrd HIEF* and *nrdDG* regulatory regions. NrdR binding motifs are enclosed in rectangular boxes. Identical bases in NrdR boxes in both strains have asterisks under them. Notice that box 2 is nearly identical in both strains (highly conserved). -10 and -35 recognition elements were reported experimentally. DnaA boxes- white letters in grey boxes. (Torrents et al., 2007)

In *nrdAB* gene promoter NrdR box 1 is overlapping the -10 promoter element essential for the start of transcription, whereas box 2 is located 16 bp downstream of the -10 element (figure 5). Box 2 is more conserved so mutation in this region causes loss binding for repressor as shown in figure 6. NrdR binds to regulatory regions in all types of reductases.
Torrents et al. found that \textit{nrdR} is transcribed constantly at similar levels at the early exponential phase of culture growth as at stationary phase. (Torrents et al., 2007)

Mutations of NrdR presented in figure 8 showed increase in expression of \textit{nrdAB} gene during early, late exponential growth and stationary phase.
Transcriptional repressor NrdR is 146-200 amino acids long polypeptide binding to NrdR boxes by zinc finger motif (~45 amino acids). The following ~90 amino acids form ATP cone domain that binds ATP/dATP. A study of NrdR protein in *Streptomyces Coelicolor* indicate that one mole of this repressor tightly binds one mole of ATP or dATP. Mutant with defective nucleotide binding amino acid sequence- ATP cone could not bind short probes containing NrdR boxes (in vitro), this indicates that in order to bind, NrdR repressor needs to change conformation provided by ATP/dATP binding. (Grinberg et al., 2009).

*S. Coelicolor* NrdR and *E.coli* NrdA ATP cone (allosteric) domain shows some homology in their crystal structure but NrdR has narrower cleft (figure 7).

![Figure 8 Homology modeling of S. Coelicolor NrdR and E.coli NrdA N terminal ATP cone domains based on crystal structure of E.coli NrdA. NrdR in contrast to NrdA has two fully conserved tyrosines (Y). Highly conserved marked amino acids were modified in Grinberg et al study. (Grinberg et al., 2009)](image)

Relative amounts of ATP and dATP in wild type NrdR were analyzed and indicating that 60% of released nucleotides is ATP where 40% is dATP. Grinberg et al., proposed that native NrdR is oligomer (octamer) that may undergo allosteric inhibition. (Grinberg et al., 2009)

Molecular concentration of dATP/dATP+ATP in *Salmonella* that has similar metabolism to *E.coli* is [0.175mM]/ [3mM] = 0.058 (Neuhard and Nygård, 1987, p.477) where NrdR binding ratio dATP/ ATP = 0.6 (Grinberg et al., 2009).
Low cellular dATP concentration that NrdR can bind indicates higher binding affinity for dATP than for ATP and this may correlate to regulation of NrdR by low levels of dATP. All of the mutants in amino acids marked in figure 7 bind mostly dATP. (Grinberg et al, 2009)

The properties of nucleotide binding domain and low cellular dATP/ATP ratios can suggest that dATP-NrdR complex is bound to DNA until dATP concentration decrease significantly and the complex dissociates.

Table 1 below presents intracellular amounts of dNTPs and ATP in *Salmonella*.

<table>
<thead>
<tr>
<th>Compound</th>
<th>µmol/g dry weight</th>
<th>Intracellular concentration (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dATP</td>
<td>0.41</td>
<td>175</td>
</tr>
<tr>
<td>dGTP</td>
<td>0.28</td>
<td>122</td>
</tr>
<tr>
<td>dCTP</td>
<td>0.15</td>
<td>65</td>
</tr>
<tr>
<td>dTTP</td>
<td>0.18</td>
<td>77</td>
</tr>
<tr>
<td>ATP</td>
<td>7.00</td>
<td>3 000</td>
</tr>
</tbody>
</table>

Table 1 Neuhard and Nygård, 1987, pg. 477.

Transcription of RNR increases if DNA synthesis is inhibited by nalidixic acid or by inactivation of temperature sensitive initiation and elongation mutants or inhibition of RNR by hydroxyurea etc. (see review Herrick et al., 2007).

**Hydroxyurea**

RNR is inhibited by hydroxyurea (HU), a free radical scavenger, which inhibits the enzymatic activity of class I RNR, that induces the expression of the *nrdAB* and *nrdHIEF* genes. (Monje-Sasas et al., 2001).

**Nalidixic acid**

Nalidixic acid (abbr. nal for the purpose of this project) is a synthetic antimicrobial compound that inhibits DNA gyrase, by targeting the enzyme’s α-subunit (Verbist L. 1986).

DNA gyrase, topoisomerase II, or simply gyrase, as the enzyme exchangably is termed, is essential for bacterial replication.
Gyrase relieves DNA-topological stress (twisting), caused by the progression of DNA polymerase III during replication, by co-ordinately introducing negative supercoils ahead of the direction of DNA-synthesis. Any inhibition of gyrase, indirectly inhibits the initiation of DNA-replication at oriC.

Addition of excessive amounts of nal, would have an inexpedient bacteriocidal effect, but in the moderate amounts used for this experiment, the effect of nal is limited to complete inhibition of DNA synthesis and partial inhibition of DNA transcription.

**Problem formulation**

The experimental part aims at answering the following question:

**Does NrdR play a role in the expression of the nrdAB gene during RNR inhibition in *E.coli*?**

Sub-questions:

- Is NrdR involved in *E. coli* cell cycle regulation?
- How is the repressor enzyme NrdR, and gene expression levels of nrdAB in *E. coli*, affected by inhibition of DNA-synthesis?

In the present study the hypothesis that NrdR is sensor for low levels of deoxyribonucleotides (dATP) will be tested. Two strains: wild type and mutant (*nrdR* gene deletion) strain will be compared. Treatment with hydroxyurea (HU) that inhibits RNR activity should lower the concentration of deoxyribonucleotides (dNTPs) and thus stimulate *nrdAB* transcription.

If NrdR is a sensor for low concentration of dATP, the wild type strain (without deletion of repressor gene) should show increase in RNR transcription in response to HU when compared with the wt strain without HU. Mutant strains with and without HU should show no difference in RNR transcription because a lack of NrdR repressor. We perform expression study for RNR using gfp fusion as a reporter. In addition, Flow cytometry was performed to see the DNA replication response to NrdR deletion in mutant strain and compare it to wild type.
Methods and Materials

Transduction

The first proposed model for monitoring expression of \textit{nrdAB} gene in order to check the hypothesis of this study was to use three different \textit{E. coli} K-12 strains where the \textit{nrdAB} promoter was fused with \textit{lacZ} as a reporter gene. The table below shows the genotypes and sources of the strains that were used in the transduction.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>BOS7</td>
<td>att\text{B::p}_{nrdA}'-lacZ</td>
<td>Tove Atlung</td>
<td>donor</td>
</tr>
<tr>
<td>MG1655</td>
<td>\text{Thi,attB::pnrdAB-\text{lacZ kan}^R}</td>
<td>Anders Løbner-Olesen</td>
<td>recipient</td>
</tr>
<tr>
<td>JW0403</td>
<td>nrd\text{R::kan}^R</td>
<td><a href="http://www.shigen.nig.ac.jp/ecoli/strain/top/top.jsp">http://www.shigen.nig.ac.jp/ecoli/strain/top/top.jsp</a></td>
<td>recipient</td>
</tr>
<tr>
<td>JD20587</td>
<td>nrd\text{R::miniTn10\text{kan, acfQ, lacZDM15, galK2, galT22, thi}}</td>
<td><a href="http://www.shigen.nig.ac.jp/ecoli/strain/top/top.jsp">http://www.shigen.nig.ac.jp/ecoli/strain/top/top.jsp</a></td>
<td>recipient</td>
</tr>
<tr>
<td>FH3964</td>
<td>\Delta nrd\text{R::cat}</td>
<td>Flemming G. Hansen</td>
<td>recipient</td>
</tr>
</tbody>
</table>

Table 2 The strains used in the transduction experiments.

For this purpose the strain BOS7 (\text{attB::p}_{nrdA}'-lacZ) was used as a donor strain to produce P1 lysate which was then used to infect the recipient strains. After the P1 lysate production, the genes taken from the donor were inserted to the recipients and selected on kanamycin (50 µg/ml) LB (Luria-Bertani) plates for kanamycin resistance. The infection by P1 lysate would insert the \textit{nrdAB} promoter, \textit{lacZ} and \textit{kan}^R fused genes in order to be able to measure the \textit{lacZ} activity. The first transduction was done on MG1655 and JW0403 strains. The infection on MG1655 was successful but JW0403 was kanamycin resistant. Then transduction was tried on FH3964 strain which resulted without success due to unknown reasons.

\footnote{The JW0403 strain was supposed to have \Delta nrd\text{R} and \text{kan}^R.}
Transformation

In order to proceed with our experimental part the transduction idea was abandoned and transformation was proposed. The transformation procedure was performed on wild type and mutant strains (*E. coli K-12*) using 3 different plasmids which genotypes are written in the table below.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pTAC5238</td>
<td>See figure 9</td>
<td>Tove Atlung</td>
</tr>
<tr>
<td>pTAC5234</td>
<td>See figure 9</td>
<td>Tove Atlung</td>
</tr>
</tbody>
</table>

**Strain**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>FH3964 (mutant)</td>
<td>ΔnrdR::cat</td>
</tr>
<tr>
<td>MG1655 (wt)</td>
<td>Thi,attB::nrdAB-lacZ kan^R</td>
</tr>
</tbody>
</table>

Table 3, shows the plasmids and strains used in the present study.

MG1655 strain was named TC5368, referred as a wild type (wt). The strain FH3964 is referred as mutant strain. These two strains were made competent by treating with CaCl₂ and the three different plasmids were transformed and the transformants selected on LB ampicillin (100 µg/ml) plates. All of the plasmids contained the *gfp* gene located downstream of the *nrdAB* promoter used as a reporter gene for measuring *nrdAB* expression in each strain. Six different combinations were measured written as following; wt/p72, mutant/p72, wt/p38, mutant/p38, wt/p34, mutant/p34. Plasmid pFH2472 was used as a control plasmid for plasmid copy number.

![Figure 9 Plasmids containing parts of nrdAB promoter region with gfp fusion are shown; pTAC5234 and pTAC5238](image-url)
**PCR (Polymerase chain reaction)**

The strains and the plasmids were checked for accuracy using 4 primers.

<table>
<thead>
<tr>
<th>primers</th>
<th>Recognition Sequence</th>
<th>Fragment length</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MG1655 (wt)</td>
</tr>
<tr>
<td>nrdR1</td>
<td>‘AAAGCAGCGAAAGCATCGATCC’</td>
<td></td>
</tr>
<tr>
<td>nrdR2</td>
<td>‘CATGTAATACTCGTCCTGCACG’</td>
<td>580bp</td>
</tr>
<tr>
<td>Gfp-5</td>
<td>‘CACATCACCATCCAGTTCCACC’</td>
<td>603bp</td>
</tr>
<tr>
<td>pBR-1A</td>
<td>‘CCGAAAAAGTGCCACCTGACG’</td>
<td></td>
</tr>
</tbody>
</table>

Table 4 Primers used for PCR, their recognition sequences and fragment length in basepairs). The mutant strain is \(\Delta nrd:cat\) so the fragment is expected to be bigger than 580bp. (Winseq program developed by Flemming G. Hansen)

Picture 1 SDS gel scan showing fragments representing wt and mutant strains with p38 and p34. \(\lambda\) Best EII marker is the MW used.
**Experimental set up**

Transformants were grown in ABTG medium with 100 µg/ml ampicillin. First the strains were grown over night on LB ampicillin (100 µg/ml) plates. The next day 10 single colonies of each culture were chosen and put into flasks with 30 ml ABTG. The cultures were grown at 37°C in shaking bath until the OD$_{600}$ reached 0.3. After they reached OD$_{600}$ of 0.3, the cultures were 100 times diluted in ABTG and again under same conditions were left to grow till OD$_{600}$ reaches 0.3. By diluting the cultures and allowing them to grow till 0.3, approximately 10 doubling in exponential phase are reached. Measurements were taken during early exponentially growing bacterial cultures because in the balanced growth the cell physiology does not change except when HU is added.

Once the cultures reached OD$_{600}$, 0.3 they were diluted 10 times in 6 flasks from which 3 flasks were treated with 5 mM hydroxyurea (HU). For the nalidixic acid assay, we used 20µg/ml (10mg/ml stock solution). All bacterial cultures were then grown under same conditions for almost 2 hours until they reached OD$_{600}$ of 0.3.

During the exponential growth of the cultures first samples (1ml) for GFP and OD$_{600}$ were taken at 30 minutes. Further samples for measurements were taken every 15 minutes of each culture. The fluorescence measurements were performed in triplets in order to gain better deviation of the results. For Flow cytometry measurements the samples were fixed and stained. (*see appendix II*)

A pilot study was initially performed in order to see whether the concentration of HU is efficient enough to give discussible results. The results from the pilot experiment revealed acceptable results and so the main experiment was performed as explained above.
Flow Cytometry

In order to measure the DNA content of the cells as well as the cell size, Flow cytometry is used. In the Apogee flow cytometer fixed and stained cells by DNA specific stain are passing through a light beam, one cell at a time, pushed by a stream of water. The parameters measured for each cell passing through the laser lights are light scatter and fluorescence. The fluorescence is proportional to the amount of DNA in the cells and the light scatter is proportional to the cell size. A standard sample which contains cells with one fully replicated chromosome (1 genome equivalence) is used in order to calculate the actual amount of DNA per cell in the sample of interest. In addition, by inhibiting the initiation of replication and cell division by addition of drugs (rifampicin and cephalexin) the number of origins per cell can be determined. (http://pcfcij.dbs.aber.ac.uk/thesis/tchap1.html)

With regards to the nalidixic acid assay, any effect hereby on DNA, should ideally be distinguishable by flow cytometric analysis, but it so happens that nal, in its relaxing effect on chromosomal supercoiling, charges the binding of the fluorescence agent EtBr, which resultingly ineffectualizes flow cytometric fluorescence analysis. Nevertheless, light scatter data from flow cytometry analysis, remains useful.

Results

This section presents the experimental data reflecting transcriptional regulation of the \textit{nrdAB} promoter. The growth of the bacterial cultures was not affected by addition of HU as can be seen in the figures in appendix III and the doubling times presented in table 6. The fluorescence from the \textit{gfp} reporter gene is a measure of expression of the \textit{nrdAB} gene, and it increases by time during early exponential phase. GFP per ml, plotted against OD\textsubscript{600}, represents fluorescence per cell mass from exponentially growing bacterial cultures. The specific fluorescence plot is used to see the effect of HU on plasmid copy number. The presentation of results for the HU assay concludes with an analysis of flow cytometry data.
Included is a section of the nalidixic acid assay, in which the chemical's effect on *E. coli* cell growth (doubling time), and *nrdAB* transcriptional levels (GFP) are investigated.

**The effect of HU on plasmid copy number**

The representation of the data in figure 10 outlines the difference of promoter activity between the cultures with and without HU. The plasmid p72 has a *mioC* promotor with defective DnaA boxes, and any addition of HU is expected to not have any influence on the promoter activity, wherefore it is used as a control for plasmid copy number. Measurements were not taken at time 0 min, but we assume that GFP/mass is the same in the beginning for both wt and mutant strains with and without HU. Initially the plasmid copy number increased in the samples treated with HU. After the first measurement the copy number declined. A small decrease was also observed in the samples in which no HU was added.

![Promoter activity of wt and mt strains with p72 with and without HU](image)

*Figure 10* Promoter activity of wt and mutant strains with p2472, with and without HU. During the exponential growth, cultures with control plasmid wt/p72 and mutant/p72, with and without HU, show a slight difference between the wild type and mutant so HU has a very small effect plasmid copy number.

The results of GFP per ml plotted against OD$_{600}$ show that the cultures containing plasmids with *nrdAB* promoter *gfp* fusion grown with HU, have a higher GFP synthesis rate than the samples without, as shown in the figures below and table 5, which means
they had a higher rate of transcription. In order to represent the data in a better way the last points of the measurements taken at 120 min are excluded.

Figure 11 represents the GFP per ml versus OD$_{600}$ of cultures with plasmid containing all promoter elements: wt/p34 and mutant/p34, with and without HU.

During the exponential growth, cultures with plasmid containing all promoter elements: wt/p34 and mutant/p34 with and without HU show increase in transcription in presence of HU for both wild type and mutant. Mutant cultures give higher values because they lack transcriptional repressor (see figure 11).

Figure 12 shows the cultures with plasmid containing only $nrdR$ binding motifs: wt/p38 and mutant/p38, with and without HU.
The cultures with plasmid containing only \( nrdR \) boxes: wt/p38 and mutant/p38 with HU and without HU show increased transcription in presence of HU during exponential growth. Mutant cultures give higher values because they lack transcriptional repressor (see figure 12).

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>p34</th>
<th>p38</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wt</td>
<td>1471</td>
<td>163</td>
</tr>
<tr>
<td>Wt+HU</td>
<td>3466</td>
<td>224</td>
</tr>
<tr>
<td>fold for wt+HU/wt</td>
<td>2.4</td>
<td>1.4</td>
</tr>
<tr>
<td>Mutant</td>
<td>4559</td>
<td>585</td>
</tr>
<tr>
<td>mutant+HU</td>
<td>5034</td>
<td>686</td>
</tr>
<tr>
<td>fold for mutant+HU/mutant</td>
<td>1.1</td>
<td>1.1</td>
</tr>
<tr>
<td>Mutant/wt</td>
<td>3</td>
<td>3.6</td>
</tr>
<tr>
<td>Mutant/wt + HU</td>
<td>1.5</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 5 shows specific activity differences in GFP transcription between samples with and without HU (horizontal), and between plasmid content (vertical).

The table 5 above shows the difference in multiplicity factor of cultures with HU compared with those without as well as the difference between the two plasmids, p34 and p38. GFP for cultures with plasmid containing all promoter elements (p34) were much higher than those with plasmid containing only \( nrdR \) boxes (p38). However the table does not show the multiplicity factor of the cultures with p72 because there is not a significant difference between the samples treated with and without HU. (see figure 10)

When comparing mutant and wild type the lack of repressor shows 3 fold higher GFP synthesis for plasmid p34 and 3.6 fold higher for plasmid p38. In the cultures with HU the mutant has 1.5 fold higher expression than wild type for plasmid p34 and 3 fold for p38.(see table 5)

Wild type strain with all promoter elements treated with HU has 15 fold higher values than plasmid containing NrdR boxes only. The same samples without HU the difference is 9 fold. For wild type strain containing p34 plasmid addition of HU make 2.4 increases whereas the p38 plasmid shows 1.4 fold increase.

Looking at the mutant from the same perspective difference is not significant. For the mutant strain containing p34 plasmid and p38 addition of HU make nearly the same increase 1.1. (see table 5)
There is a difference between the same strain and plasmid number (wt/p38 and wt/p38+HU) treated with and without HU. The samples treated with HU show an increase in the promoter activity by 1.4 fold due to the inhibition of RNR enzyme and thus stimulation of its expression.

**Cell cycle of wild type and mutant cultures**

To look at cell cycle of bacterial strains flow cytometry was performed. This section starts with brief explanation of cell cycle in fast growing bacteria as a short introduction to flow cytometry results. Result section compares cell cycle of wild type and mutant.

**Nalidixic acid assay GFP results**

In the following section we will look at the results of adding a 10μg/ml solution of nalidixic acid, to four different *E. coli* cultures consisting of the wildtype and mutant *nrdR* strains, each transformed with the p34 and p38 plasmids.

![GFP fluorescence vs. cell growth - wild type](image)

Figure 13. On the left: GFP fluorescence vs. cell growth for both wildtype transformants, with and without addition of nalidixic acid. On the right: an enlarged image of the wt/38 curves from the left graph.
The above figure illustrates the difference in transcriptional levels of the wildtype strain, with and without the addition of nal. Firstly a distinct difference in GFP levels can be noticed between the two transformants; the intact plasmid p34, has the highest GFP levels, regardless of whether or not nal was added. Secondly, addition of nal does not result in an increase of transcriptional levels, for both plasmids; an increase is only observed in the case of p34, whereas the opposite effect is discovered with regards to p38, as can be clearly seen in the blown up image on the right of the above figure. Addition of nal increases GFP 3.5-fold for p34, whereas it leads to a ca. 40% decreases in p38.

![GFP fluorescence vs. cell growth - mutant](image1.png)

**Figur 14.** On the left: GFP fluorescence vs. cell growth for both mutant transformants, with and without addition of nalidixic acid. On the right: an enlarged image of the mt/38 curves from the left graph.

The above GFP vs. OD graphs, deal exclusively with the mutant strain, i.e. we investigate the effect of nalidixic acid on both plasmids, in the absence of NrdR. As in the case with the wildtype strain, transcriptional levels are higher for the p34 plasmid, compared to the the p38 plasmid, regardless of whether or not nal was added.
In the left window, it can be clearly seen that addition of nal induces a (1.3-fold) stimulation of promotor activity in the p34 transformant, whereas for p38, the effect is insignificant.

The table below gives an overview of the different transcriptional slopes from fig. 14, and how they mutually compare (multiplicity factors).

<table>
<thead>
<tr>
<th>mutually compared slopes from GFP per ml vs. OD_600</th>
<th>plasmid type</th>
<th>p34</th>
<th>p38</th>
<th>(p38)/(p34)</th>
</tr>
</thead>
<tbody>
<tr>
<td>strain</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>wt</td>
<td></td>
<td>1471</td>
<td>144</td>
<td>0.10</td>
</tr>
<tr>
<td>wt+</td>
<td></td>
<td>5229</td>
<td>91</td>
<td>0.02</td>
</tr>
<tr>
<td>(wt+)/wt</td>
<td></td>
<td>3.55</td>
<td>0.63</td>
<td>0.18</td>
</tr>
<tr>
<td>mt</td>
<td></td>
<td>4559</td>
<td>585</td>
<td>0.13</td>
</tr>
<tr>
<td>mt+</td>
<td></td>
<td>5934</td>
<td>649</td>
<td>0.11</td>
</tr>
<tr>
<td>(mt+)/mt</td>
<td></td>
<td>1.3</td>
<td>(1)</td>
<td>(0.77)</td>
</tr>
</tbody>
</table>

Table 6 GFP slopes compared
Cell cycle of fast growing bacteria

![Diagram of cell cycle in fast growing bacteria]

**Figure 13** Cell cycle in fast growing bacteria. C corresponds to DNA synthesis and D corresponds to cell division. Time of C and D is estimated as the minimal time needed. G refers to the generation time - doubling time; green (origins), yellow (replication complex), purple (terminus) (Atlung, 2004)

Cell cycle overlaps in fast growing bacteria by inducing new synchronous initiations before one cell cycle finish (cell division) and that’s the reason why generation time is shorter than the sum of DNA synthesis - C and cell division - D. The level of free DnaA is responsible for synchronized initiations where one origin can initiate only once per cell cycle. New origins are hemimethylated and newly synthesized DnaA boxes bind free DnaA molecules. (Atlung, 2004)
Flow cytometry analysis

The figure below represents DNA per cell mass in cultures without rifampicin and cephalexin. Results from the samples treated with rifampicin and cephalexin are not shown because DNA per cell mass was the same.

![DNA per cell mass](image)

**Figure 14 FL/LS versus time (min) represents DNA/mass for wt and mutant strains**

In the figure above HU decreases DNA/cell mass in wild type and mutant cultures. The HU has the highest effect in mutant and wt strains at 60min. As the time is increasing the effect is lower so the DNA/cell mass is increasing.

In the analysis, data for population of cells is calculated and their distribution monitored by Winflow program for samples taken directly from the exponentially growing cultures. The data from these measurements will give an idea about the elevation of the dNTP pools in both wt and mutant strains with and without HU.

Presented figures are from samples where initiation and cell division is inhibited by rifampicin and cephalexin respectively. Existing origins are allowed to finish so the genome equivalents (*table 6*) reflects the number of origins at the time of cell birth. (*figure 13*)
In the wt cultures there is an increase in the number of origins and decrease in DNA/mass at 60min, in the samples with HU. The majority of the cells are with 8 chromosomes due to slow replication.
The histograms in figure 16 are representing DNA distribution in mutant strain. These distributions are used as a control for HU effect on the DNA content in the cells. As seen in the figure above at the same time cells with 4 chromosomes are the most abundant in both samples and the population of the cells with 2 and 8 chromosomes is similar. The genome equivalents for these samples are 4.7 and 4.9 respectively and the samples without HU replicate a bit faster. Generally, the replication in the mutant strains is faster because C+D period is shorter than the wt. (see table 6)

**Cell cycle properties of exponentially growing cultures**

<table>
<thead>
<tr>
<th>strain</th>
<th>g.e, tD, C+D</th>
<th>wt/p72</th>
<th>mutant/p72</th>
<th>wt/p38</th>
<th>mutant/p38</th>
<th>wt/p34</th>
<th>mutant/p34</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+HU</td>
<td>+HU</td>
<td>+HU</td>
<td>+HU</td>
<td>+HU</td>
<td>+HU</td>
<td>+HU</td>
</tr>
<tr>
<td>g.e for 60min</td>
<td>5.7</td>
<td>6.6</td>
<td>4.7</td>
<td>4.9</td>
<td>4.8</td>
<td>5.9</td>
<td>4.5</td>
</tr>
<tr>
<td>tD (min)</td>
<td>35</td>
<td>34</td>
<td>34</td>
<td>34</td>
<td>29</td>
<td>29</td>
<td>30</td>
</tr>
<tr>
<td>C+D (min)</td>
<td>88</td>
<td>93</td>
<td>76</td>
<td>78</td>
<td>66</td>
<td>75</td>
<td>65</td>
</tr>
</tbody>
</table>

Table 7: The genome equivalents (ori/cell) for 60min, doubling time (tD) and the sum of the time between initiation and termination of a round of replication, C, and termination of chromosome replication till next cell division, D, are presented. C+D is calculated by the following formula: \(\text{ori/cell} = \frac{2^{C+D}}{tD}\).

The table above shows the genome equivalents/cell for wt and mutant strains from flow cytometry analysis. The wt and mutant with p72 are control for the HU effect on the plasmid copy number should be the most relevant to look at. The wild type strain where the regulation of \(nrdAB\) operon is fully functional shows an increase in genome equivalence (5.7-6.6 g.e.) in cultures with HU. This corresponds to an increase in (C+D). The mutant on the other hand, does not show a significant difference in the samples treated with and without HU (4.7-4.9 g.e.).
In order to present cell cycle by flow cytometry diagrams, wild type and mutant cultures with plasmid containing mioC promoter-p72 were chosen. The reason for that is that the cell cycle of these cultures is not affected by the presence of plasmid. Plasmids with promoter elements could saturate some fraction of regulatory proteins like DnaA so less can act on the chromosome.

Discussion

Many studies have been carried out in E. coli revealing that when the DNA/mass ratio in is abnormal, nrdAB gene expression is increased. Under normal conditions this occurs approximately during initiation. However transcription of RNR increases if DNA synthesis is inhibited by nalidixic acid, inhibiting RNR by HU or by inactivation of temperature sensitive initiation and elongation mutants etc. (Herrick et al., 2007).

Recently, a few research papers have been published proposing a new regulation of the RNR gene expression connected to nucleotide pool sizes. The new protein firstly identified in Streptomyces (Borokov et al., 2004), NrdR, acts as a strong repressor. Binding of dATP/ATP to NrdR regulates binding to a tandem 16-bp NrdR-box sequences located in the promoter region of nrdAB operon. (Grinberg et al., 2009).

In the present study the transcriptional regulation of nrdAB gene in E.coli was analyzed through the new regulator NrdR. An increase in the rate of nrdAB gene expression by inhibition of RNR by HU was observed, however higher in wt than in the mutant. Therefore the presence of the NrdR repressor is an essential factor in the regulation of nrdAB gene expression. nrdAB operon is regulated independently of its location, on chromosome or plasmid (see review Herrick et al., 2007) so measuring GFP from plasmids is relevant.
**Comparison of wt and mutant strains**

In this study the GFP measurements (see table 1) reveal 3 fold lower *nrdAB* promoter activity in wild type than in mutant (*nrdR* gene deleted) which shows the effect of NrdR repression. However, the level of GFP at the OD$_{600} = 0.3$ was 2.4 higher in the mutant than in the wild type. Torrents et al., used *nrdR* deletion-substitution mutants (see figure 7) to study whether, NrdR regulates the transcription of *nrdAB* in *E.coli*. It has been found that the transcription is increased by 1.5 fold in a supposed null mutant (ATP binding domain and zinc finger motif defect) compared to wt at early exponential phase. In Torrents et al., study the cell cultures for their measurements were grown in LB medium and measured at early exponential phase OD$_{550} = 0.4$ (Torrents et al., 2007). The measurements in our study were done in early exponential phase (until OD$_{600}$ reach 0.3) and bacteria growth in ABTG medium. The differences in experimental performance might be the reason for variation in our and Torrents et al., results. However, in both studies NrdR negatively regulates *nrdAB* expression.

In addition to our GFP measurements the Flow cytometry results reveal that wild type strains are replicating slower (based on C+D period) than the mutant and have more origins per cell. In wild type the NrdR contribute to the overall control of the amount of RNR expressed which keeps constant dNTPs pool in the cell. Since elongation is limited by the amount of dNTPs (see review Herrick et. al.), we assume that the reason for faster replication of the mutant is increased synthesis of dNTPs that are used for faster DNA replication (see table 6). Higher levels of dNTPs than normal are due to higher level RNR.

Moreover allosteric regulation in the primary regulatory site of RNR can contribute to the amount of active RNR present in the cell depending on dATP level. Since dATP is used during the DNA synthesis at exponential growth the active RNR is present in the cells.
• **Wild type and mutant with p34 and p38**

To have closer look at NrdR function we narrowed down the regulatory region to NrdR boxes and some DnaA binding motifs only (p38) and compared to fully functional promoter (p34). In wild type strains p38 NrdR protein is present and binds to the promoter on the plasmid controlling GFP expression. On the other hand in mutant strain where gene coding for NrdR is deleted, synthesis of GFP is by 3.6 fold higher than in the wild type and in plasmid p34 the increase is by 3 fold. *(see table 5)* The repressor has stronger effect when only NrdR binding boxes are present. This data shows that presence of other activators negatively affect the repression of NrdR. This might be due to the modulating repression by activation or protein-protein interaction.

• **The effect of HU on plasmid copy number**

Hydroxyurea was used to inhibit RNR and therefore decrease dNTPs. As a result nrdAB promoter activity is induced. Since we are using plasmids with nrdAB gfp fusion the effect of HU on plasmid copy number has to be investigated. Therefore plasmid with mioC promoter (FHp2472) with inactivated DnaA boxes is used as a plasmid copy number control.

Initially all the cultures start from a same point at time 0 min and measurements were not taken at that time so we assume that the plasmid copy number initially increased with HU (p72) and then decrease. However, in p72 the cultures without HU had almost constant fluorescence (see figure 10). This shows that there is a small effect of HU on cells’ plasmid copy number.

The strains with plasmid p34 and p38 grown with HU might have faced the same effect of HU and decrease in the plasmid copy number by the time, giving lower fluorescence. If there was not a decrease in the plasmid copy number the promoter activity of strains with HU would have shown slightly higher promoter activity.
- **Effects of HU on nrdAB expression**

  In wild type +HU the expression goes up by factor of 2.4 because RNR is inhibited so the dNTPs respectively decrease and then the repressor dissociates from DNA. The synthesis of GFP between mutant strain with and without HU showed no significant increase in the culture with HU (1.1 fold). When comparing wt and mutant with HU there is 1.5 fold increase observed in mutant strain. The multiplicity factors described in this context represent strains with p34 which is considered as wt promoter.

  Since there is still difference in gfp synthesis in wt and mutant p38 with and without HU where NrdR boxes and DnaA box are present then DnaA is the activator that contributes to this expression. The role of DnaA as an activator hasn’t been exactly defined and so far only three cases have been published proposing that it acts as an activator for *nrdAB*. Løbner-Olesen et al., 2008; Tuggle and Fuchs, 1986; Augustin et al., 1994. However, the effect on the mutant remained the same as p34 (1.1 fold). The comparison of wt and mutant with p38 in the presence of HU showed 3 fold increase.

  The promoter type in wild type play a role in response to HU and 15 fold contribute to the highest GFP value for wt/p34 +HU. In contrast, there was 9 fold increase between plasmids in the absence of HU in the same strain. When HU effect is used to compare wt/p34 and wt/p38, the results show that wt/p34+HU increase greater than wt/p38+HU with difference of 2.4 and 1.4 folds as mentioned above. This also shows that the greater increase in wt/p34 +HU might be caused by interactions of NrdR with other proteins bound upstream of the promoter that may influence its affinity for nucleotides as it has been proposed by Grinberg et al., 2008. If NrdR is the only factor in the negative regulation of the transcription, addition of HU shouldn’t show any effect in both plasmids in mutant cultures. However, the observed difference might be due to another protein acting as a repressor.
To conclude from the above discussed results, NrdR is the protein responsible for sensing low dNTP pool. According to our hypothesis in presence of HU NrdR dissociates from the promoter region allowing transcription of nrdAB so there shouldn’t be a difference between wt with HU and any mutant because there is no repression in either case.

- **Flow cytometry analysis reveal that HU decreases DNA/mass**

In general Hydroxyurea decreases the DNA/cell mass which can be observed from figure 14. The rate of the DNA synthesis depends on the active RNR and addition of HU inhibits RNR activity. Therefore dNTPs go down and elongation gets slower but cell mass continues to increase. As a direct result the number of replication forks per chromosome increase (see review Herrick et al., 2007).

In wt as a result of DNA/mass decrease in HU samples the g.e./cell increases from 5.7 to 6.6 and the DNA synthesis decrease as well. In contrast in mutant DNA/mass per cell also decreases with addition of HU but the g.e./cell and DNA synthesis is the same with and without HU.

We presume that initially the amount of active RNR is higher in mutant than in wt as corroborated by the GFP results. Since the same concentration of HU was used, the inhibition in mutant strain is lower as shown in table 5. This is also observed in figure 14, where the DNA/mass per cell is lower in the wt than the mutant.

The effect of hydroxyurea is decreasing as the cell senses low dNTPs and nrdAB transcription goes up. At 60 min dNTP level is already low and sensed by the repressor in wt cultures. A signal has already been sent to the nrdAB promoter region that RNR concentration in the cell has to be increased in order to keep balanced dNTP pools. However, HU still inhibits RNR but at a lower degree. The dNTPs are still lower then optimal because inhibition is also seen at 120 min. Consequently DNA/mass per cell in samples with HU is increasing over time.
• The role of NrdR in RNR transcription

The inhibition of RNR causes decrease in the dNTP pools which can result in replication anomalies and mutations because the rate of DNA synthesis at the replication fork depends in the amount of active RNR (Herrick et al., 2007). The prokaryotes struggle against the decrease of dNTP pools by increasing the rate of activation of nrdAB genes by increasing the transcription of RNR. This explains the higher promoter activity in the presence of HU in the present study.

These results point out the role of NrdR as a repressor that inhibits transcription of nrdAB promoter. The increased transcription in the mutant strain is due to the deletion of the gene coding for NrdR repressor which is absent to sense low dNTP pool. It has been proposed that NrdR has higher affinity for dATP when it’s present at low concentrations which reflect the size of dNTP pool. The proposition is based on the study by Grinberg et al., where it has been found that NrdR binds ATP/dATP via its ATP cone domain which contributes to a conformational change and binds to 16 bp sequences in the nrdAB operon by zinc finger motif. (Grinberg et al., 2009)

Inhibition of DNA-synthesis affects nrdAB expression

• Inhibition of DNA synthesis increases the repressive efficacy of the enzyme NrdR on the nrdAB promoter.

If an unbalancing of the dNTP/NTP ratio in E. coli is registered by any repressor or activator of nrdAB, a change in the genes transcriptional levels can be followed by GFP measurements.

With the wt/p38 culture, as the only exception, addition of nal., resulted in a marked increase in GFP. Nal addition had a directly opposite effect in the case of wt/p38, which strongly indicates that inhibition of DNA synthesis leads to a strengthening of NrdRs potency as a repressor for nrdAB.
As already mentioned in the introduction, it has been experimentally confirmed that the dATP-form of NrdR, in contrast to the ATP-form, binds more efficiently to its respective boxes on the \textit{nrdAB} promotor. Thus, since the p38 plasmid only contains NrdR boxes and a few DnaA boxes, one can infer the possibility that a fall in GFP observed for \textit{wt/p38} could be the result of increased levels of dATP in the cell.

Intuitively, it seems very plausible that dNTP pools would immediately increase – at least for a short span of time – following the inhibition of DNA synthesis by nal. But given that RNR has a an extremely sensitive allosteric feed-back mechanism (Larsson et al 2004, and Jordan et. all 1998) , increases in dATP/ATP, would immediately be registered by RNR, and set a stop to any overproduction of dATP by RNR, thus not increasing NrdR's dATP form. In order to draw conclusion here, one would have to compare the sensitivities of NrdR and RNR to changes in dATP/ATP. This goes beyond the scope of the current project.

- **Inhibition of DNA synthesis reduces cell size and density**

The sharp-drop followed by a slow-rise pattern for all LS data, suggests the following explanation. Inhibition of gyrase leads to a partial inhibition of RNA polymerase, besides completely inhibiting replication (Lee, 1976). Both polymerases suffer from the inhibition of gyrase, but gyrase is only irreplaceable in the case of replication, whereas transcription – with it's 20-fold lower nucleotide processing rate – can do with topoisomerase I, which only removes positive supercoils, in contrast to gyrase which can introduce negative supercoils, and change linking number by 2.

Partial inhibition of RNA polymerase, results in less mRNA transcript, and thus less cell protein which is reflected in the reduced LS values within the first hour of nal addition. This finding corresponds well with the doubled doubling times. Another factor to consider, regarding the decreased LS values, is the fact that the septa-division phase eventually disappears, since new rounds of replication are stunted by the addition of nal. Inhibition of DNA synthesis prior to the end of a round of replication , inhibits cell division. Alternatively, inhibition of cell division after the termination of a round of
replication, but prior to the initiation of another round of replication, does not inhibit cell division (Clark, 1968)

Even though cell-division eventually levels out completely OD continues to increase. This is due to that all cells eventually become arrested in a filamentous state, where they continue to elongate but do not divide. Hypothetically, the only growth-parameter would be the amount of DNA already present in the cells, which would determine how long they could grow, given inexhaustible space and nutrients.

- **Inhibition of DNA synthesis has an overall stimulating effect on nrdAB expression, which can be definitely separated from the regulatory role of NrdR.**

The wt/p34 assay ascertains this, and it is further corroborated by wt/p38 (which shows that NrdR by itself, only plays a repressive regulatory role)

Addition of nal leads to an 88% greater GFP increase in wt/p34 compared to wt/p38. This strongly suggests that activator sites are primarily present in p34, whereas the promotor stretch of p38 mainly contains binding-sites for repression. Two sets of unaccounted for palindromic sequences in the promotor of nrdAB, revealed by means of DNA sequencing, further supports the existence of these unidentified activator compounds.

- **Addition of nal has a 2.8-fold greater stimulating effect on nrdAB expression, in the presence of NrdR.**

This is explained by the fact that nrdAB expression is already very high, due to the lack of a repressor, thus addition of nal – which induces a unknown stimulating factor – has little additional stimulating effect of transcriptional levels. Nevertheless, mt/p38 + nal, tops with the highest transcriptional level.
addition of nalidixic acid has a lesser effect on wt/p34 compared to mt/p34

This makes sense, considering that the mutant is acting constitutively, thus already having very high levels of GFF prior to nal addition. Addition of nal only has a meagre 30% percent increase in the mutant, compared to a 355% increase in the wildtype.

Hypothetical calculations based on table 6 (GFP slopes):

\[
\frac{(mt+)}{(mt)} = 1.3: 30\% = \text{nal-induced increase in GFP ascribable to any other promotor stimulating factors, other than derepression of NrdR. This calculation does not though take into consideration, what the loft for } nrdAB \text{ promotor activity might be. In fact the following calculation shows that 30\% can impossibly be the case, suggesting that GFP levels of near 6000, are close to the maximum promotor capacity.}
\]

\[
\frac{(wt+)}{(wt)} = 3.55: 355\% = \text{total nal-induced increase in GFP. And since NrdR only has a repressive effect (assuming that it doesn't interact with other activator factors in the p34 extra stretch), one can conclude that DNA-synthesis inhibition, massively stimulates one or more compounds which act as activators on } nrdAB. \text{ Otherwise the following nonsense calculation would be true: } 355\% - 30\% = 322\%; \frac{322\%}{355\%} = .91 = \text{fraction of total nal-induced promotor stimulation, that can be ascribed to derepression of NrdR.}
\]
Reference:


Websites:

http://pcfcij.dbs.aber.ac.uk/thesis/tchap1.html
### Appendix

#### Appendix I

Summary of antibiotics used for the bacterial culture and their mechanism

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Process inhibited</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Nalidixic acid</td>
<td>Supercoiling</td>
<td>DNA gyrase</td>
</tr>
<tr>
<td>2 Rifampicin</td>
<td>Initiation of transcription (RNA synthesis)</td>
<td>RNA polymerase</td>
</tr>
<tr>
<td>3 Chloramphenicol</td>
<td>Translation (protein synthesis)</td>
<td>Ribosomes</td>
</tr>
<tr>
<td>4 Cephalexin</td>
<td>Cell division</td>
<td>Septum formation</td>
</tr>
<tr>
<td>5 Hydroxy Urea (HU)</td>
<td>DNA synthesis</td>
<td>RNR</td>
</tr>
</tbody>
</table>

Table 8 Summary of antibiotics used for treatment of bacterial strains for flow cytometry and gfp measurements and their function. 2,3 (Nelson and Cox, pg.1006,1066);1,4 (genetics lab manual 2009;Dept. of Systems and Models); 5 (Slater M. , 1973)
Appendix II

Materials and Methods

SUPPLEMENT 1– bacterial Growth medium recipes

ABTG

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile water</td>
<td>900ml</td>
</tr>
<tr>
<td>A10</td>
<td>100ml</td>
</tr>
<tr>
<td>1M MgCl$_2$</td>
<td>1ml</td>
</tr>
<tr>
<td>1M CaCl$_2$</td>
<td>0.1ml</td>
</tr>
<tr>
<td>0.01M FeCl$_3$</td>
<td>0.3ml</td>
</tr>
<tr>
<td>4mg/ml Thiamin (B1)</td>
<td>0.4ml</td>
</tr>
<tr>
<td>20% glucose</td>
<td>10ml</td>
</tr>
<tr>
<td>20% Casamino acids</td>
<td>50ml</td>
</tr>
</tbody>
</table>

Table 9 ABTG supplement

A10 salts: per 1L

\[(NH_4)_2SO_4 20 g\]
\[Na_2HPO_4 60 g\]
\[KH_2PO_4 30 g\]
\[NaCl 30 g\]
Add Milli Q H$_2$O to a volume of 1 L. Autoclave, control pH to a constant value of 7.1.

LB (rich medium)

10 g tryptone
5 g yeast extract
5 g NaCl
11 H$_2$O

Production of P1 phage lysate by the plate method:

Use an old P1 lysate as starter lysate.
Grow an overnight culture of the donor strain in 5 ml LB (+ antibiotics or thymine if appropriate) at appropriate temperature (37 C or 30 C for Ts strains)
Put the LB plates in 37 el. 42 C incubator to prewarm them.
Melt 50 ml LB topagar (0.7% agar). Only use the microwave with less than half full flasks and remove the cap from the flask. Else use a boiling waterbath. Add 10 ml LB and CaCl$_2$ to 10 mM (needed for adsorption of P1 phage).

Add 5 ml topagar to 10 ml Nunctubes in a heatingblock (or waterbath) at 45 C.

Make appropriate (e.g. 10$^{-1}$, 10$^{-2}$, 10$^{-3}$, 10$^{-4}$, 10$^{-5}$) dilutions in LB of the starter P1 lysate.

Mix 100 µl of each phage lysate dilution and 200 µl o.n. bacterial culture.

Make 1 tube with only bacterial culture (control for OK growth).

Add the mixture of phage and bacteria to a tube with topagar, mix by rolling the tube gently and pour the contents onto a prewarmed LB plate.

Leave the plates for ½ hr to allow the topagar to solidify, put the plates into a plastic bag and incubate bottom up over night at appropriate temperature (37 C or 30 C for Ts strains).

Next day choose the best plate(s) i.e. a plate with almost but not quite total lysis. Harvest the phages by scrabing the topagar into a 9 ml polypropylene centrifuge tube, wash the plate with 2-5 ml LB. Add MgCl$_2$ to approx. 10 mM (stabilizes the P1 phages) and ½ ml CHCl$_3$ (in the hood) to lyse remaining bacteria and to sterilize the phage lysate. Mix by sucking up and down in a pipette. Leave for ½-1 hr

Spin down agar and cell debrises, 10 min at 10.000g in Ole Dich centrifuge.

Transfer the supernatant to a fresh centrifuge tube. Add ½ ml CHCl$_3$. Add a tight fitting cap to the tube and leave overnight at 4 C.

Next day remove cap and centrifuge again.

Transfer supernatant to a sterile glass tube with screw cap. Label with P1(strain) and date.

Keep the lysate at 4 C. (

NB! Never freeze a phage lysate, limit exposure to room temperature and do not whirlimix.
**P1 transduction**

Grow o.n. culture of recipient strain (+ antibiotics or thymine if appropriate) at appropriate temperature.

Add CaCl$_2$ to 10 mM

Make $10^0$, $10^{-1}$, $10^{-2}$, $10^{-3}$ dilutions in LB of P1 donor lysate.

Mix 100 µl of each phage dilution with 200 µl o.n. culture. remember control without phage.

Incubate at 37 C for 20 min.

**With selection for antibiotic resistance**

Add 1 ml LB with 1% citrate (1M NaCitrate is ca. 30%). Incubate with shaking for 1-2 hrs at appropriate temperature, normally 37 C.

Spin down cells 2 min 10.000, resuspend pellet in 1ml LB + citrat (NB only LB if selection for kana$^R$). Spin down again.

Resuspend i 200 µl LB + citrat. (NB only LB if selection for kana$^R$)

Plate on LB + kanamycin.

Control for sterility: Plate 100 µl undiluted P1 lysate directly on selective plate.

Incubate o.n. at appropriate temperature, normally 37 C.

Restreak an appropriate number of colonies (from the plate with the most dilute lysate that gave transductants) on selective plate + streaking on appropriate check plates.

If the first restreaking plate shows signs of residual phages (lysis in thick of streak) restreak once more.
Preparation of competent cells by Ca\(^{++}\) procedure using Ole Dich centrifuge

- A fresh overnight culture of the host strain is diluted 1:25 in LB medium and incubated in a shaking water bath until OD\(_{450}\) reaches 1. This takes 1 to 2 hr, depending on the strain and the growth temperature.

- Harvest 6x8 ml culture in 9 ml tubes by centrifugation in the Ole Dich refrigerated centrifuge at 8000 g for 2 minutes. The centrifuge and rotor should preferentially be prechilled to 4°C.

- Wash the cells with 6x6 ml ice-cold 10 mM MgSO\(_4\). Start by adding 1 ml and resuspend cells with a short shaking on the whirly mixer, add remaining 5 ml and centrifuge again at 8000 g for 2 minutes. Pour off the liquid.

- Resuspend the cells gently in 6x1 ml ice-cold 50 mM CaCl\(_2\) using the pipette. Take care not to disrupt the cells, they are quite fragile now. Collect the cells into 2-3 tubes, fill up with ice-cold 50 mM CaCl\(_2\) to 8 ml pr tube and incubate at 0°C for 30 minutes.

- Harvest the cells (8000 G for 2 minutes). Resuspend the cells as above in 2 ml ice-cold 50 mM CaCl\(_2\) and incubate at 0°C for 60 minutes or longer. The cells are now competent. They may be kept for several days in the refrigerator.

Transformation of chemically competent cells (Ca method)

- Place (labelled) E-tubes in an ice bath. Always include a no DNA control (and an uncut plasmid control for transformation of ligation mixtures)

- Add 20 – 200 μl\(^g\) of cells to each tube

- Add 0 – 20 μl of DNA (DNA max 1/10 volume of cells)

- Incubate on ice for 30 min or longer time

- Heat shock by incubation at 42 C for 5 min (heating block or water bath)

- Place tubes at room temperature and add 100 – 1000 l of LB (5 times volume of cells)
- Incubate at appropriate temperature – normally 37°C for 30 min – 2 hrs§
- Plate on dried LB plates containing appropriate antibiotic. Concentrate cells by short centrifugation if volume > 250 μl. Incubate plates at appropriate temperature o.n.

# Ca++ cells: use 50 μl for transformations with ccc plasmid DNA

use 200 μl for transformations with ligation mixtures

§ Time depends on antibiotic 30 -60 min OK for ampicillin.

**PCR**

(Biometra Personal Cycler™) Running program 7
- Start procedure: denaturation of DNA 2 min at 94°C
- Cycle procedure: denaturation 30 sec at 94°C
- annealing 60 sec at 57°C (gfp-5 and pBR-1A) and 54°C (nrdR1 and nrdR2)
- elongation 1 min at 72°C
  - for 25 cycles
- Stop procedure: finishing all elongation 10 min at 72°C
- Standby (optional) 999 min at 4°C

**PCR reaction**

Make a 10-fold dilution of the two overnight cultures E-buffer (for example 90 μL E-buffer + 10μL culture).

Place the tubes on ice and make a master mix for a number of necessary reactions, adding the components in the order given in the Table below. The volumes are very small, so make sure you have something in the pipette tip, and that it is emptied, change the tip for each pipetting to avoid contamination of the solutions. At the end assemble all drops at the bottom of the master mix tube by a 2 sec spin in the small tabletop centrifuge.

Then add 24μL mastermix per PCR tube, and 1μL of the different templates. Keep the samples on ice until the PCR machine is started.
### Stock solutions

<table>
<thead>
<tr>
<th>Stock solutions</th>
<th>Concentration in stock solution</th>
<th>µl per reaction</th>
<th>µl for 11 reactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile water</td>
<td></td>
<td>14</td>
<td>154</td>
</tr>
<tr>
<td>dNTP mix</td>
<td>2.5 mM of each</td>
<td>2</td>
<td>22</td>
</tr>
<tr>
<td>Reaction buffer</td>
<td>10X</td>
<td>2,5</td>
<td>27.5</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>25 mM</td>
<td>2</td>
<td>22</td>
</tr>
<tr>
<td>nrdR-1</td>
<td>20 µM</td>
<td>1</td>
<td>11</td>
</tr>
<tr>
<td>nrdR-2</td>
<td>20 µM</td>
<td>1</td>
<td>11</td>
</tr>
<tr>
<td>Gfp-5</td>
<td>20 µM</td>
<td>1</td>
<td>11</td>
</tr>
<tr>
<td>pBR-1A</td>
<td>20 µM</td>
<td>1</td>
<td>11</td>
</tr>
<tr>
<td>Fermentas Taq polymerase</td>
<td>5 units/µl</td>
<td>0,2</td>
<td>2.2</td>
</tr>
</tbody>
</table>

*Table 10 PCR master mix*

### Agarose gel electrophoresis

The DNA samples are mixed with DNA sample buffer before loading on the gel. This is a Tris, HCl buffer containing EDTA to stop all enzyme reactions by complexing the Mg\(^{++}\), sucrose to make the sample “heavy” so it will drop to the bottom of the well and bromphenol blue which allows you to see where you put the sample and to follow the progression of the electrophoresis.

The DNA in the gel is visualised by staining with ethidiumbromide which fluoresces orange when intercalated into DNA and irradiated with UV light.

### Preparation of agarose gel

When making, for instance, a 1.0 % agarose gel the following is mixed in a 250-500 ml Erlenmeyer flask.

1.0 g agarose
100 ml 1 x TBE buffer

The mixture is boiled well (3 - 5 minutes) in the microwave oven until the gel solution is clear.
The gel mixture is cooled to 60° - 80°C before addition of 10 µl 4 mg/ml ethidium bromide after which the mixture is poured into a mould and slotformers are placed. After 45 minutes the gel is set and the slotformers are removed.

Running buffer:

- 80 ml 10 x TBE buffer
- 720 ml milli-Q water
- 8 µl 4 mg/ml ethidium bromide

**Flow Cytometry**

In order to measure the DNA content of the cell per cell mass, samples were taken for Flow Cytometry measurements at 60 min of the exponentially growing cell cultures and at the end when the cells reached OD$_{600}$ of 0.3. The samples were fixed (see detailed procedure below) addition of 77% ice cold ethanol and left over night at 4°C The following day the samples were stained by taking 150 µl of samples in new E-tubes and spin down 4 min at 10000g. The pellet was resuspended in 140 µl staining solution and left in dark on ice for minimum 30 min and then the samples were run in the Flow Cytometer (Apogee Flow Systems Ltd.). Samples treated with rifampicin and cephalexin were left overnight in water bath to complete cell division. They were also fixed and stained the same way and run in the flow cytometer. 45ml ABTG + amp with and without HU (5mM) were prepared first and placed in water bath at 37°C. To each 45ml of ABTG+ amp. and ABTG + amp. +HU 5ml of cell cultures were added. Strains TC5368 and FH 3964 with plasmids: pFH 2472, p5234, p5238.

Samples for FC 0.5ml of cells were taken after 60 min every 30 mi to E-tubes placed on ice. Rif+ceph samples were taken every at 60 min and at the end.

1ml cell culture+2ml ABTG+amp+60µlRif+ceph were incubated over night at 37°C in glass tubes.
FIXATION:

Note: Leave the samples treated with Rifampicin+Cephalexin in the fridge till the next day (Wednesday) and then do the fixation.

1. 0.5ml cell culture in each E-tube on ice!
2. Spin down by centrifugation 5min at 10000g 4 degrees.
3. Put them back on ice and remove the supernatant
4. Resuspend in ice cold Tris Buffer pH=7.5 (it is in our fridge) by whirlmixing
5. Add 1ml of 77% Ethanol and mix.
6. Leave them in the fridge till measurement.

Staining: (to be done on the day of measuring)

Stain the solutions by first putting them on ice (from the fridge)

1. Whir mix and put 150μl of the cells in new E-tubes (on ice).
2. Spin down 4 min at 10000 4 degrees.
3. Remove supernatant and resuspend in 140μl staining solution. Whirl mix and leave them in DARK till measurement. They should stay at least 30min in DARK
4. MEASURE (check FC machine for water and turn on 15min before running the samples)

GFP samples

1ml of bacterial cells samples were taken every 30 mi in E-tubes. To GFP samples 50 μl chloramphenicol (4mg/ml) was added to inhibit protein synthesis.

Plate with 96 wells was used where 100μl from each GFP sample (previously whirl mixed) were placed in 3 wells (triplets). Measurements were done by GFP (Biotek Synergy HT) apparatus.

Reference:
-Tove Atlung lab recipes
Appendix III – Cell growth

A. The hydroxy urea assay.

The 2hr-duration growth curves of the cultures, with and without the addition of 5mM HU, are represented by plotting OD$_{600}$ against time on a logarithmic scale. The results show that all the cultures had a normal exponential growth, with doubling times around 30 min. In the figures below it isn’t easy to distinguish though, because all the cultures had almost the same growth wherefore they overlap when plotted in a logarithmic scale. The purpose of these graphs is, to show that during the experiment the cultures had a similar exponential growth.

Figure 17, shows the normal growth of the samples wt/p72 and mutant/p72 with and without HU. OD$_{600}$ plotted against time.

In the figure above it is not possible to see distinguish the overlapping growth curves from one another, because the cultures had almost the same exponential growth as explained in the previous text.
Figure 18, represents the growth of the cultures wt/p38 and mutant/p38 with and without HU.

In the figure it’s not clear to see that mutant/p38, with and without HU, had the shortest doubling time of 29 minutes.

Figure 19, shows the exponential growth of cultures wt/p34 and mutant/p34, with and without HU.

Fig. 19 shows that mt/p34, with and without HU, had the longest doubling time of 36 minutes.

The cultures wt/p34 and mutant/p34, which were grown under similar conditions with and without 5mM HU, showed the same exponential growth. A difference was
observed between the 30min. doubling time of wt/p34 without HU, and the 35min. doubling time of the culture wt/p34 with HU (figure 3).

B. The nalidixic acid assay

![cell growth vs. time - wildtype](image1)

![cell growth vs. time - mutant](image2)

**Figure 20.** Exponential growth curves of the cultures wt/p34, wt/p38, mt/p34, mt/p38, with and without the addition of nalidixic acid

Fig. 20 clearly shows that addition of nalidixic acid to the given cultures has a marked effect on cell growth rate. For both strains, addition of nalidixic acid approximately halves their growth rates; in other words it doubles their doubling times. Addition of nal, appears to have a slightly less diminishing effect on growth rate in the case of mt/34.

The table below gives an overview of the changes in culture doubling times, as a result of nalidixic acid treatment.
### Appendix IV – Flow Cytometry Light-Scatter

Regardless of which culture one picks out from the table, light-scatter (LS) data shows that nalidixic acid results in a distinct drop in LS, especially within the first hour after addition. After 1.5h, LS gradually steps up, and increases for all 2h data available.

LS information is composed of forward-scatter and side-scatter, which respectively correlate with cell volume, and the inner complexity of the cell.

<table>
<thead>
<tr>
<th>time (min)</th>
<th>wt/34</th>
<th>wt/38</th>
<th>wt/34 +</th>
<th>wt/38 +</th>
<th>mt/34</th>
<th>mt/38</th>
<th>mt/34 +</th>
<th>mt/38 +</th>
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</thead>
<tbody>
<tr>
<td>60</td>
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<td>75</td>
<td>38</td>
<td>76</td>
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<td>90</td>
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<td>75</td>
<td>53</td>
<td></td>
<td>79</td>
<td></td>
<td>47</td>
<td>65</td>
</tr>
</tbody>
</table>

Table 12 nalidixic acid assay light-scatter values

---

<table>
<thead>
<tr>
<th>culture type</th>
<th>control culture</th>
<th>culture +</th>
<th>control culture/(culture +)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mt/p38</td>
<td>29</td>
<td>59</td>
<td>2,0</td>
</tr>
<tr>
<td>mt/p34</td>
<td>36</td>
<td>55</td>
<td>1,7</td>
</tr>
<tr>
<td>wt/p38</td>
<td>30</td>
<td>56</td>
<td>1,9</td>
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<tr>
<td>wt/p34</td>
<td>30</td>
<td>61</td>
<td>2,0</td>
</tr>
</tbody>
</table>

Table 11 doubling time for the cultures shown in fig.20

---

**Doubling times $t_D$**

<table>
<thead>
<tr>
<th>culture type</th>
<th>control culture</th>
<th>culture +</th>
<th>control culture/(culture +)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mt/p38</td>
<td>29</td>
<td>59</td>
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