Antisense regulation of cell division in *Escherichia coli*

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Abstract

BACKGROUND: FtsZ is an essential cell division protein in bacteria. It is involved in the initiation, timing, and frequency of cell divisions. Consequently it is subject to strict regulation, including transcriptional and post-transcriptional control and protein inhibition. Regulation of ftsZ expression by an antisense RNA has also been proposed. Dewar and Donachie suggested that there is a gene stfZ encoded by the antisense strand of DNA at the fisA-ftsZ intergenic region in the mra gene cluster of the Escherichia coli chromosome. Multiple copies of the DNA fragment containing the stfZ gene block cell division. Since an RNA transcribed from stfZ would be complementary to the ribosomal binding site of ftsZ, it has been proposed that the product of stfZ is an antisense RNA that controls translation of ftsZ.

RESULTS: To see if there are other antisense genes in the mra gene cluster of the E. coli chromosome, we looked for antisense promoters and terminators in the region. While no promoters have been identified, all the DNA fragments that have been tested had strong termination activity. Northern blotting, RT-PCR, and primer extension experiments confirm that there is an RNA produced from the antisense strand of DNA at the fisA-ftsZ gene junction of E. coli cells. RT-PCR experiments indicate that the StfZ RNA is at least 423 nt long. Overexpression of an artificial RNA containing a part of the StfZ sequence lowers FtsZ concentration in cells, prevents FtsZ ring formation, and inhibits cell division. Experiments with fusion constructs show that the overexpressed RNA acts on the ribosomal binding site (RBS) of fisZ. Other experiments using the fusion constructs indicate that the expression from the RBS of fisZ is strongly dependent on the growth phase of cells.

CONCLUSIONS: The antisense RNA StfZ is produced in E. coli cells. It is very likely that StfZ controls translation of fisZ, and that it does this by binding to the RBS of the fisZ mRNA.
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Declaration

I declare that the composition of this thesis was all my own work, except where stated.

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Abbreviations

b: base(s)
bp: base pair(s)
cAMP: cyclic adenosine 3',5'-monophosphate
DAPI: 4,6-Diamidino-2-phenylindole
DNA: deoxyribonucleic acid
dsRNA: double stranded RNA
_E. coli_: Escherichia coli
EDTA: ethylenediaminetetraacetic acid
IPTG: isopropyl β-D-thiogalactoside
kbp: kilo base pair(s)
MU: Miller unit(s)
nt: nucleotide
o/n: over night
OD₆₀₀: optical density at 600 nm wavelength
ORF: open reading frame
PCR: polymerase chain reaction
PTGS: posttranscriptional gene silencing
RBS: ribosomal binding site
RNA: ribonucleic acid
RNAi: RNA interference
RT-PCR: reverse transcription coupled PCR
SD: Shine-Dalgarno
siRNA: short interfering RNA
siRNP: small interfering ribonucleoprotein
X-gal: 5-bromo-4chloro-3indolyl-β-D-galactopyranoside
1 Introduction

1.1 E. coli cell division

Escherichia coli are facultatively anaerobic rod-shaped Gram-negative bacteria that usually live in the intestinal tracts of animals. They multiply by dividing at the middle of the long axis of a cell into two daughter cells. The division proceeds by the circumferential invagination of the cytoplasmic membrane accompanied by the synthesis of peptidoglycan and invagination of the outer membrane. Septum formation is mediated by a septal ring, a cytoskeletal structure that is associated with the cytoplasmic membrane at the site of division. It is assembled before the start of invagination and remains associated with the leading edge of the septum until septal closure. The division process is coordinated with DNA replication and segregation [for reviews, see ref. 84, 125, 181].

1.1.1 The septal ring

The E. coli septal ring is a multimeric complex that consists of at least nine essential proteins: FtsA, FtsI, FtsK, FtsL, FtsN, FtsQ, FtsW, FtsZ, and ZipA [25, 38, 60, 62, 117, 119, 174, 210, 297]. The first recognisable event in E. coli cell division is the formation of an FtsZ ring at the middle of a cell [32]. FtsZ is a cytoplasmic GTPase [66], structurally very similar to eukaryotic tubulin [91]. It is likely that FtsZ nucleates at a single point and polymerises bidirectionally to form a ring [3]. This ring contracts during cell division while maintaining a position at the leading edge of the invaginating septum [32]. To form a septal ring, FtsZ recruits other cell division proteins to the division site. The proteins assemble in a strict order: first FtsZ, then ZipA and FtsA independently of each other, followed by FtsK, FtsQ, FtsL, FtsW, FtsI, and FtsN — the recruitment of each of these proteins depends on localisation of the previous one to the septum.

The FtsZ ring forms at the division site independently of any other proteins that have been tested so far [2, 54, 111, 147, 167, 194, 224, 297, 298].

ZipA is anchored in the cytoplasmic membrane and is thought to be involved in the assembly of the septal ring by linking the FtsZ ring to the membrane. In vitro, ZipA
has also been shown to promote and stabilise FtsZ protofilament assembly [237]. It interacts directly with FtsZ [118, 122, 176]. ZipA requires FtsZ to be localised, but does not require FtsA, FtsQ, FtsL, or FtsI [55, 111, 120, 167].

FtsA is a cytoplasmic protein, but is also found associated with the membrane. It belongs to the actin family of proteins, and is structurally homologous to actin and heat-shock protein Hsc70 [289, 34a]. FtsA is able to bind ATP and can be isolated in either phosphorylated or non-phosphorylated form [250], but the biological significance of this is not yet known. FtsA can interact with itself and this interaction is probably important for its function [315]. FtsA binds FtsZ directly [299], and its localisation to the division site depends on FtsZ, but not FtsL or FtsN [1, 54, 111].

FtsK is a huge protein consisting of an N-terminal domain with several membrane-spanning regions, a proline-glutamine rich domain, and a C-terminal domain with a nucleotide-binding consensus sequence [26]. It has several functions that allow FtsK to couple chromosome segregation with cell division [15, 37, 165, 267, 297]. The N-terminus of FtsK is essential for E. coli cell division and for its membrane localisation [87, 297, 316]. The C-terminus has been shown in vitro to use ATP hydrolysis to translocate DNA [15]. It also promotes complete Xer recombination between dif sites that is necessary to resolve chromosome dimers after chromosome replication [15, 19]. FtsK is a homologue of the Bacillus subtilis SpolIIIE protein that is required for chromosome segregation during spore formation [20]. FtsK expression increases as a part of the SOS response [297]. Its localisation to the division site depends on FtsZ, ZipA, and FtsA, but not FtsQ or FtsI [224, 297].

FtsQ is a bitopic membrane protein with a short cytoplasmic N terminus, a single membrane-spanning segment, and a periplasmic domain [55]. The cytoplasmic domain may be important for cell division, but the membrane-spanning segment probably has no role in FtsQ function other than acting as an anchor [45, 55, 116]. The periplasmic domain of FtsQ determines its localization [45, 55]. The function of FtsQ is not yet clear. FtsQ localisation to the division site depends on FtsZ, FtsA, and FtsK, but not FtsL, FtsI, or FtsN [54, 55].
FtsL is also a bitopic membrane protein with a short cytoplasmic N terminus, a single membrane-spanning segment, and a periplasmic domain. The cytoplasmic domain and the membrane-spanning segment have both been shown to be essential for cell division [116]. The membrane-spanning segment and the periplasmic domain of FtsL are required for its localization to the division site [110]. The periplasmic domain has a repeated motif characteristic of leucine zippers and may be involved in FtsL multimerisation [110]. The function of FtsL is still unknown. Its localisation to the division site depends on FtsZ, FtsA, FtsQ, and FtsK, but not FtsI or FtsN [54, 111].

FtsW is a membrane protein homologous to the RodA protein of *E. coli* (important for the cell shape determination) and to the SpoVE protein of *Bacillus subtilis* (involved in spore formation) [138]. It has been suggested that the main function of FtsW and its homologues is to recruit specific transpeptidases (FtsI in case of *E. coli* FtsW) to the correct location in the cell [194]. The *E. coli* *ftsW* gene has two overlapping open reading frames that encode two possible FtsW peptides – FtsWL (large) and FtsWS (small). FtsWL is the major protein produced in *E. coli* cells, but FtsWS is also sufficient for cell division, if overexpressed [38, 147]. FtsW localisation to the division site depends on FtsZ, FtsA, FtsQ, and FtsL, but not FtsI [194].

FtsI (also known as penicillin-binding protein 3, PBP3) is another bitopic protein with a short cytoplasmic domain, a single membrane-spanning segment, and a large periplasmic domain. The periplasmic domain of FtsI catalyses transpeptidation and is involved in the synthesis of septal peptidoglycan. The cytoplasmic domain and the membrane-spanning segment are also required for cell division [116]. FtsI localisation to the division site depends on FtsZ, FtsA, FtsK, FtsQ, FtsL, and FtsW, but not FtsN [54, 194, 298, 307].

FtsN is yet another bitopic protein with a short cytoplasmic amino terminus, a single membrane-spanning domain, and a large periplasmic carboxy terminus [63]. The periplasmic domain determines the localisation of FtsN [2]. The cytoplasmic and transmembrane domains are probably not required for the function of FtsN other than to anchor the periplasmic domain [63]. FtsN localisation to the division site has been shown to depend on FtsZ, FtsA, FtsQ, and FtsI [2, 298].
1.1.2 Location of the septal ring

An *E. coli* cell normally divides in the middle to make two similar daughter cells. One of the most interesting questions about such division is how the middle of a cell is recognised. How does the FtsZ protein know where to start the septal ring formation? It appears that in *E. coli*, the FtsZ ring positioning is controlled by two systems that prevent the FtsZ ring formation in cell areas other than the midcell: the Min protein system and the nucleoid occlusion.

The MinC, MinD, and MinE proteins of *E. coli* are necessary for accurate positioning of the cell division site. It has been found that *E. coli* mutants lacking the Min proteins form FtsZ rings and divide either at the middle of the cell (as normal) or at the poles (forming small chromosomeless ‘minicells’, Fig. 1) [68]. The MinCD protein complex was shown to inhibit the assembly of the FtsZ ring [31, 223]. In the absence of MinE, MinCD inhibits cell division both at the cell poles and the midcell [68]. MinC is the inhibitor in this system. MinC N-terminal domain prevents FtsZ polymerisation, while the C-terminal domain is required for interaction with MinD [133]. MinC does not affect the GTPase activity of FtsZ [59, 131]. It requires MinD for activation (this might be achieved simply by concentrating MinC at its site of action) [70] and positioning in the cell [67, 132]. When expressed at high levels, MinC can act as a division inhibitor even in the absence of MinD [70]. MinE is a suppressor of the inhibitory activity of MinCD. It prevents MinCD from acting at the middle of the cell while permitting it to block division at the cell poles [68]. MinD is a peripheral cytoplasmic membrane protein. It binds ATP and has ATPase activity important for the MinC-dependent division inhibition [67]. MinD recruits both MinC and MinE to the membrane [70, 233]. The binding of MinD makes MinC sensitive to the suppression of the inhibitory activity by MinE, since it has been shown that in the absence of MinD, overexpressed MinC inhibits cell division regardless of MinE [70]. It was shown that the Min proteins can potentially localize anywhere in the cell [58a].
The MinCDE system ensures that the FtsZ ring does not form at the cell poles. For this purpose the inhibitor MinC needs to be concentrated at the cell poles and absent from the midcell. This is achieved in *E. coli* cells through an amazing mechanism that involves rapid oscillation of MinC from pole to pole [132, 234] (Fig. 2). The process requires both MinD and MinE proteins. In the absence of MinD, MinC is a cytoplasmic protein [132]. If MinD is present but MinE is absent, MinC is distributed homogeneously over the membrane [132]. Since MinC positioning in a cell depends on MinD, it is not surprising that MinD was also found to be distributed homogeneously over the entire cytoplasmic membrane in the absence of MinE [245], and to oscillate in a MinE-dependent manner [235]. On the other hand, MinD itself is required for MinE positioning [233]. In the presence of MinD, MinE has been found to form a ring at or near the middle of a cell, independently of the FtsZ ring [233]. This ring was later shown to be a very dynamic structure. MinE localisation changes in a rapid cycle during which most of the protein assembles into a ring near the middle of a cell and moves towards a cell pole where it dissipates; then a new ring again assembles near the middle of the cell and moves to the opposite pole [121]. The MinE localisation cycle is tightly coupled with the oscillations of MinCD, and the MinE ring probably corresponds to accumulation of MinE at or near the dynamic border between the areas of the cytoplasmic membrane containing or lacking MinD.
It has been suggested that MinE stimulates MinD disassembly in a wave-like manner, starting from midcell and progressing towards one of the poles, thus keeping the middle of the cell free of MinCD [121].

Fig. 2. Localisation of Min proteins in *E. coli* cells. MinD recruits MinC to the cytoplasmic membrane. The MinE protein moves in a wave-like fashion from midcell towards the cell poles in an alternating sequence and stimulates release of the MinCD complex from the membrane thus inducing MinCD to oscillate between the opposite cell poles. This mechanism ensures that the average concentration of division inhibitor MinC is lowest at the midcell. MinC and MinD are shown here as a complex during all the stages of oscillation, though it is not known yet if MinC stays associated with MinD after MinD leaves the membrane. Adapted from ref. 121.

The exact mechanism for the Min protein oscillations is still unknown, but several models have been proposed that attempt to explain these oscillations mathematically. Meinhardt and de Boer show that several assumptions, such as that MinD self-assembles on the membrane and recruits MinC and MinE, that the local accumulation of MinE is generated by a pattern formation reaction that is based on local self-enhancement and a long range antagonistic effect, and that MinE displaces MinD from the membrane causing its own local destabilisation and shift toward higher MinD concentrations, are sufficient for generating oscillations observed in *E. coli* cells [190a]. Another model also uses a simplified system, assuming a cell to be cylindrical, of a constant length, with homogeneous contents. The molecules are presumed to move in purely diffusive manner, except for the following assumptions: membrane-bound MinD molecules tend to aggregate; MinD increases MinE affinity...
for the membrane; MinE increases MinD detachment rate. Like in the model of Meinhardt and de Boer, it was found that such preset rules are sufficient to generate MinE and MinD oscillations similar to the experimentally observed ones [151].

Consistent with the above models, the ratio of MinD and MinE proteins in a cell is important for the frequency of MinCD oscillations [235]. The length of oscillation cycles affect the efficiency of Min system in inhibiting FtsZ ring formation at the cell poles. Oscillation cycles of between 30 s and 2 min have been found to be compatible with normal cell division. Moderate overexpression of MinD lengthens the oscillation cycle and allows minicells to form, presumably because MinCD stays too long in one cell pole thus allowing the FtsZ ring formation to start in the opposite cell pole. This phenotype is suppressed by simultaneous overexpression of MinE [235].

Thus it appears that an *E. coli* cell has three potential division sites, and their usage is regulated by the Min proteins. How are these potential division sites determined? There is a possibility that an altered membrane composition marks these sites (the poles would probably be marked in the same way as the midcell because the poles are the previous division sites of the cell). To investigate this, the phospholipid composition of *E. coli* division sites has been examined. It was done by determining the phospholipid content of minicells that are formed after cell division at the cell pole in *minCD* mutants. It was found that minicells have increased cardiolipin and decreased phosphatidylglycerol levels when compared with normal cells [150]. However, neither of these phospholipids is necessary for cell division or cell growth [148], so it was proposed that the change in specific phospholipid levels is needed to facilitate membrane curvature at the division site [150].

Another explanation for the three potential division sites could be that the rest of the cell is not available for the FtsZ ring formation because of interference by chromosomal DNA. This mechanism is known as nucleoid occlusion. It has been found that *E. coli parC* mutants lacking normal chromosome segregation make FtsZ rings only in nucleoid-free areas of the cells [271]. In the absence of MukB, a protein probably involved in nucleoid condensation, the negative effect of the nucleoid on the FtsZ ring formation is partially suppressed [319]. When *E. coli* cells lack the Min
protein system in addition to having faulty chromosomal segregation, they form multiple FtsZ rings in nucleoid-free regions [318]. This indicates that FtsZ has the ability to form rings at all positions along the cell length, not just at the midcell and cell pole sites [318].

Therefore it is possible that the division site in *E. coli* cells is selected in the following way: after chromosome replication and segregation the two nucleoids confine the FtsZ ring formation to the middle of the cell and the cell poles. The MinCDE system inhibits the FtsZ ring formation at the cell poles. This leaves only the middle of the cell open for the FtsZ ring to form and start the cell division.

### 1.1.3 Timing of the septal ring formation

For correct cell division, the septum dividing the two daughter cells should form after the completion of chromosome replication. It has been shown that in *E. coli* cells grown under steady-state conditions, the assembly of the FtsZ ring is initiated approximately simultaneously with the termination of DNA replication [78]. However, it is not yet known what is the mechanism that links septum formation with chromosome replication. It has been proposed that *ftsZ* transcription is linked to the rate of chromosome replication [166]. This could provide a way to coordinate chromosome replication with cell division in different growth conditions that might alter DNA replication time. No signal has yet been found to link termination of chromosome replication with cell division. Initiation of chromosome replication has been shown not to be necessary for timing of cell division [115].

### 1.1.4 Genes and regulation of their transcription

Most of the genes involved in *E. coli* cell division are organised in gene clusters, the largest of these being the *mra* cluster (for murein region A; it is also known as *dcw* gene cluster, for division and cell wall) at the 2 min region of the *E. coli* chromosome. It contains the essential cell division genes *ftsL, ftsI, ftsW, ftsQ, ftsA*, and *ftsZ*, and also genes required for cell wall peptidoglycan synthesis (Fig. 3).
Fig. 3. The mra cluster. Products of the genes ftsL, ftsI, ftsW, ftsA, ftsQ, and ftsZ are essential for septum formation [25, 38, 60, 117, 174, 226]. Products of the genes murE, murF, murD, murG, murC, and ddlB are involved in the biosynthesis of bacterial cell wall peptidoglycan [161, 183, 191, 193, 321]. The product of the envA gene is important for cell permeability and the cell separation process [215, 270]. T, transcription terminator. Promoters that have been discovered so far are shown beneath the genes.

The gene order in the mra cluster is conserved in bacteria, even though it is not essential for gene function [212]. Analysis of the mra cluster gene arrangement in different bacteria shows that the relationships between the genes are not random and might reflect the coupling between growth and division of bacteria [278a].

The mra cluster has several promoters but only one transcription terminator. It is located just downstream of envA and has a sequence of a typical rho-independent terminator [21]. Promoter P_mra, at the very beginning of the cluster, is essential for transcription of the first nine genes of the cluster, and also contributes to transcription of the genes located further downstream, including the ftsZ gene [123, 192]. There are seven promoters characterised in the ddlB-envA region of the mra cluster [4, 79, 100, 243, 270] (Fig. 3), but it has been shown that about two thirds of transcriptional activity entering the ftsZ gene originates upstream of the ddlB gene [71, 99]. This region still needs to be examined to find the exact locations of promoters and to determine if and how they are regulated.

The regulation of transcription from the mapped promoters in the ddlB-envA region is quite complex. Promoters P_{ftsQ2}, P_{ftsQ1}, P_{ftsZ4} and/or P_{ftsZ3}, and P_{ftsZ2} have been shown to be regulated inversely by growth rate [4, 82, 100, 105, 263, 266]. Such regulation allows the cells to produce a nearly constant amount of protein per cell at all growth conditions [4, 79, 266], which is important since an E. coli cell needs to make only one septum per cell to divide correctly. The mechanism by which growth rate affects transcription from these promoters is not yet clear. The concentration of two transcriptional regulators, stationary phase sigma factor σ^S and stringent response effector nucleotide ppGpp, increases during transition to stationary phase.
For this reason, they have been considered as possible regulators of the promoters $P_{ftsQ2}$, $P_{ftsQ1}$, $P_{ftsZ4}$, $P_{ftsZ3}$, and $P_{ftsZ2}$. $\sigma^S$ has been shown to increase transcription from $P_{ftsQ1}$ during the stationary phase [17, 263], but none of the other promoters of the $ ddlB-envA$ region were affected by either $\sigma^S$ or ppGpp [17, 141, 211, 228, 263, 266].

Transcription from the promoter $P_{ftsQ2}$ has been shown to be positively regulated by SdiA [300]. SdiA facilitates the RNA polymerase binding to $P_{ftsQ2}$ and so stimulates transcription [314]. On the other hand, SdiA has also been found to inhibit transcription from the $P_{ftsQ1}$ promoter, probably by competing with the RNA polymerase in binding to $P_{ftsQ1}$ [314]. SdiA has sequence similarity to a family of transcriptional regulators that respond to extracellular signal molecules derived from homoserine lactone [105]. These molecules are released by bacteria and accumulate in the medium, and so can act as quorum sensing signals. The quorum sensing system in bacteria controls gene expression in a cell density dependent manner. Expression of $sdiA$ has been shown to be negatively regulated by a factor released into the growth medium by $E. coli$ [105]. This indicates that cell-to-cell signalling may be important for regulating cell division events in $E. coli$.

The promoter $P_{ftsA}$ has been found to be positively regulated by the RcsB protein [49, 109]. RcsB is a part of a complex signalling system that activates transcription of genes involved in synthesis of capsular polysaccharide colanic acid [269]. It has been shown that RcsB can be phosphorylated by a sensor molecule and is more active in phosphorylated form [109]. Phosphorylation occurs through a phosphorelay signal transduction pathway RcsC (sensor kinase) $\rightarrow$ YojN (phosphotransfer intermediate) $\rightarrow$ RcsB (response regulator) [278]. It is not yet clear what signals RcsC responds to, but it has been reported that RcsC and RcsB are essential for osmotic induction of colanic acid synthesis [265]. Also, the $RcsC \rightarrow YojN \rightarrow RcsB$ system has been shown to respond to chlorpromazine-induced stress [58]. It is possible that alterations of the cell envelope induce a signal recognised by the RcsC protein [58].

There is also some evidence that the nucleotide ppGpp might be a positive regulator of cell division. It has been reported that mutants lacking ppGpp form filaments [313]. Also, cell division inhibition by inactivation of PBP2 can be alleviated either by overproduction of FtsZ or by increasing ppGpp concentration in the cells [291]. It
is not yet known if this effect is achieved by direct transcriptional regulation of cell division genes by ppGpp. As mentioned earlier, promoters $P_{ftsZ2}$, $P_{ftsQ1}$, $P_{ftsZ4}$, $P_{ftsZ3}$, and $P_{ftsZ}$ are not regulated directly by ppGpp, but not much is known about promoters upstream of $ddlB$, and these could be the targets of ppGpp regulation. Alternatively, ppGpp might activate transcription of other genes whose products affect expression or function of cell division proteins.

Experiments using $ftsZ::lacZ$ fusion on the chromosome of $E. coli$ have shown that both nalidixic acid treatment and thymine starvation significantly repress transcription from promoters contributing to $ftsZ$ expression. This is true when all upstream promoters or just promoters from the $ddlB-ftsZ$ region are present [166]. Both nalidixic acid and thymine starvation block chromosome replication. This suggests that chromosome replication might be linked to cell division through regulation of transcription of cell division genes. Some experiments show that the FtsA protein might also have a role in the possible connection between DNA replication and cell division [285a].

The other three genes encoding septal ring proteins, $ftsK$, $zipA$, and $ftsN$ are located at 20, 54.5, and 88.5 min of the $E. coli$ chromosome, respectively [62, 85]. The $minC$, $minD$, and $minE$ genes make a cluster at 32 min of the $E. coli$ chromosome.

The $ftsK$ gene is transcribed from two promoters. Only one of them, $P_{ftsK1}$ (previously known as $dinH$), is essential. $P_{ftsK1}$ is regulated by a repressor LexA and is induced by the SOS response [85]. Nothing is yet known about transcriptional regulation of the $zipA$, $ftsN$, and $min$ genes of $E. coli$.

### 1.2 FtsZ

FtsZ is the most highly conserved bacterial division protein. As described in the previous section, it plays a major role in $E. coli$ cell division. Until recently, it has been thought that FtsZ is conserved in all eubacteria and archaea. However, several prokaryotes have now been found to lack FtsZ analogues in their genomes [28, 92, 143, 145, 268]. *Chlamydia pneumoniae* and *C. trachomatis* are both constitutive
parasites that live within membrane-bound compartments of their mammalian host cells, and it has been proposed that they use host division proteins [92]. On the other hand, a thermophilic archaeon *Aeropyrum pernix* and a mycoplasma *Ureaplasma urealyticum* are both capable of dividing in laboratory culture even though their genomic sequences have no recognisable *ftsZ*. This indicates that some prokaryotes use an FtsZ-independent cell division mechanism.

The FtsZ protein has an important role in division of plastids in plants and algae. This is not surprising as plastids are believed to have evolved from photosynthetic cyanobacteria that have been engulfed by early eukaryotic cells [112, 189]. Dividing plastids form a PD (plastid-dividing) ring. It consists of two or three rings localised at the plastid constriction site: an inner ring on the stromal surface of the inner membrane, an outer ring on the cytosolic surface of the outer membrane, and sometimes a third ring in the intermembrane space of the plastid [152, 198]. Homologues of the bacterial FtsZ proteins have been shown to be essential for chloroplast division [220]. Higher plants have two families of FtsZ homologues, FtsZ1 and FtsZ2. The FtsZ proteins from both these families are encoded by nuclear DNA but are targeted to chloroplasts [104, 187]. They have been shown to form rings in the stromal compartment at the plastid constriction site [187, 201, 292]. There is evidence that the plastid FtsZ ring is distinct from the PD ring. During division of a red alga *Cyanidioschyzon merolae*, the FtsZ ring forms and dissociates earlier than the PD ring [197]. It has also been shown that the outer PD ring of *C. merolae* does not contain FtsZ [196]. This indicates that two different systems are involved in division of plastids, one of them originating from bacteria and based on FtsZ, and the other probably originating from the eukaryotic cells and based on the PD ring formation.

Another eukaryotic organelle, mitochondria, have also originated from engulfed bacteria [112]. Despite this, no *ftsZ* analogues have been found in the completed sequences of *Saccharomyces cerevisiae* and *Caenorhabditis elegans* nuclear or mitochondrial genomes [219]. Mitochondrial division in most eukaryotic organisms requires dynamin [for reviews, see ref. 64 and 92]. Dynamins are also known to be involved in endocytosis [128]. They can form rings and spirals similar to those of
FtsZ [92], but the dynamin spirals are usually located on the outside of the constricting membrane [33], while the FtsZ rings form on the inside. Even though dynamin involvement in mitochondrial division appears to be widespread, it seems that division of the endosymbiotic ancestors of mitochondria was based on the FtsZ protein. Recently, two algae species have been found to produce FtsZ analogues that are targeted to mitochondria and are probably involved in mitochondrial division [22, 275]. These mitochondrial FtsZ proteins are closely related to the FtsZ proteins of \( \alpha \)-proteobacteria, the bacterial predecessors of mitochondria [113].

1.2.1 FtsZ structure and function

The formation of the FtsZ ring at the middle of the cell is the first observable event in \( E. \) \( \text{coli} \) cell division. FtsZ is responsible for determining the plane of division in the cells. It has been shown that in \( E. \) \( \text{coli} \) \( ftsZ26 \) mutant, the mutated FtsZ protein forms spirals instead of rings, which leads to formation of spirally invaginating septa [3]. To fulfil its function in cell division, FtsZ has to recognise the midcell, polymerise there into a ring structure, recruit other cell division proteins, and, possibly, provide a mechanism for constriction of the septum.

The FtsZ protein is a homologue of tubulin, a eukaryotic cytoskeletal protein. The crystal structure of FtsZ from \( \text{Methanococcus jannaschii} \) has been solved and has been shown to be very similar to that of tubulin (Fig. 4) [91, 169, 214]. FtsZ is a GTPase [66, 238]. It binds GTP at the highly conserved tubulin signature nucleotide binding motif [209]. The \( \gamma \)-phosphate of bound GTP affects the conformation of loop T3 of the FtsZ protein, and consequently GTP hydrolysis causes conformational change in FtsZ [83]. Similar GTP hydrolysis-dependent conformational change is thought to occur in tubulin [213].

Another FtsZ region important for interactions with GTP is loop T7 and the start of helix H8, both located on the opposite side of the protein from the GTP binding site [170]. Like in tubulin heterodimers and microtubules, FtsZ loop T7 region interacts with GTP bound to another FtsZ monomer. Together with the nucleotide binding site on an adjacent FtsZ monomer it probably forms an active site for GTP hydrolysis. This is supported by mutagenesis experiments showing that several mutations in loop T7 region inhibit GTP hydrolysis by FtsZ [172, 253, 254, 299].
FtsZ self-assembles into polymers both *in vivo* and *in vitro* [142, 205, 207]. At the moment, most of the knowledge about the structure of the polymers comes from *in vitro* experiments. It has been shown that in the presence of GTP, FtsZ polymerises into protofilaments [39, 93, 205, 206]. GTP binding, but not hydrolysis, is necessary for polymerisation [205, 206]. It is still not clear if there is a critical concentration of FtsZ required for polymerisation to start [206, 244, 251]. In polymers, one Mg$^{2+}$ is bound per each FtsZ monomer [242]. Mg$^{2+}$ is not required for polymerisation, but is necessary for GTP hydrolysis, and so for the dynamic behaviour of the FtsZ polymers [207]. As mentioned above, the active site for GTP hydrolysis is probably formed by interaction of two adjacent FtsZ monomers during protofilament assembly. Such active site formation is probably followed by immediate GTP hydrolysis, since several studies suggest that FtsZ polymers contain mainly GDP and inorganic phosphate [252, 255]. On the other hand, other experiments indicate that nucleotide turnover throughout an FtsZ polymer is very fast, and the polymer
contains mostly GTP nucleotides [195a]. FtsZ polymers can be stabilised by addition of GTP-gamma-S – a nucleotide that mimics GTP but blocks further growth of the polymers. Addition of GDP leads to disassembly of pre-formed polymers [206, 255]. It was therefore suggested that FtsZ polymers are stabilised by a small fraction of GTP containing FtsZ monomers that may form a GTP cap similar to the one in microtubules [255].

D.-J. Scheffers and A.J.M. Driessen proposed a model in which FtsZ polymerisation closely resembles polymerisation of tubulin [251]. According to the model, as GTP-containing FtsZ monomers bind to each other, an active GTPase site is formed, and the GTP of one of the subunits is immediately hydrolysed. Afterwards, GTP-containing FtsZ monomers bind mainly to the plus end of the growing polymer (the end containing a nucleotide-binding site). This leads to formation of an FtsZ protofilament with most of its subunits containing GDP, and a subunit at the plus end containing an exposed GTP. The FtsZ protofilament is stable as long as a GTP-containing monomer is present at the plus end. Addition of a GDP-containing monomer leads to destabilisation of the polymer. The possible lateral interactions between the FtsZ protofilaments are not considered. This model assumes that most of the nucleotides bound by the FtsZ polymers are GDP, which contrasts with the data from experiments by Mingorance et al. [195a].

The FtsZ protofilaments can exist in two conformations, straight and curved [93, 171, 206]. The curved protofilaments can appear as minirings when adsorbed to a cationic lipid monolayer [173]. The protofilament conformation is thought to depend on the nucleotide bound by FtsZ. GTP has been shown to favour the straight conformation (a non-hydrolysable GTP analogue or GTP in conditions not allowing its hydrolysis by FtsZ were used), while GDP has been shown to favour the curved conformation [173]. It was proposed that the transition from straight to curved protofilaments may provide a mechanism by which the energy of GTP hydrolysis could be used to generate force for the constriction of the FtsZ ring in bacterial cell division [173].

At high concentrations of cations, such as Mg$^{2+}$, Ca$^{2+}$, or DEAE-dextran, FtsZ protofilaments can further assemble into sheets, bundles, and tubes [93, 170, 173,
It is not known yet which of these structures, if any, is relevant in vivo. Indication that such higher order FtsZ polymer structures may be important in bacterial cell division comes from experiments showing that a cell division protein ZipA also induces bundling of FtsZ protofilaments [118, 237]. Furthermore, it has been shown that at the conditions causing FtsZ protofilament bundling the rate of GTP hydrolysis by FtsZ is reduced, resulting in higher FtsZ polymer stability [207, 251].

The ability of ZipA to stabilise FtsZ protofilament assembly and to further organise FtsZ protofilaments is similar to the that of MAP-Tau protein in microtubule formation [237]. Other cell division proteins may also be important for FtsZ polymer formation in vivo, and more experiments are needed to determine this.

FtsZ has been shown to interact directly with two cell division proteins involved in septum formation, ZipA and FtsA [119]. Both ZipA and FtsA bind to the C-terminus of FtsZ, but in different ways, since several FtsZ mutations have been found to affect their binding differently [118, 122, 176, 203]. Also, FtsZ can probably interact directly with MinC [140, 223], a repressor protein from the Min system involved in selection of a division site (see section 1.1.2 on page 10). Another protein that can interact with FtsZ is SuIA (SfiA), an inhibitor of cell division induced in response to DNA damage [134]. SuIA has been shown to inhibit polymerisation and the GTPase activity of FtsZ [142, 286]. There is also evidence that FtsZ interacts with molecular chaperone proteins DnaK and HscA [46, 188, 287]. Both DnaK and HscA co-sediment with FtsZ polymers [141, 287]. Mutations in DnaK cause severe defects in cell division that can be alleviated by overexpression of FtsZ [46]. Cells with mutated HscA form filaments and have highly reduced numbers of FtsZ rings, while hscA conditional knockout cells have abnormal localisation of FtsZ under non-permissive conditions. Overexpressed DnaK can partially complement the HscA mutation [287].

1.2.2 Regulation of FtsZ expression and function

The concentration of FtsZ protein in an E. coli cell is important for determining frequency and timing of division, it can also affect the position of the division septum [30, 60, 280, 301]. Thus the expression of FtsZ has to be tightly regulated.
ftsZ is the penultimate gene in the mra gene cluster (Fig. 5). The cluster has several promoters, but transcription is stopped at the only terminator located downstream of the envA gene [21]. Transcriptional regulation has been examined mainly for the promoters in the ddlB-ftsZ region: P_{ftsQ2}, P_{ftsQ1}, P_{ftsA}, P_{ftsZ3}, and P_{ftsZ} (see section 1.1.4 on page 14). Several of these promoters are regulated inversely by growth rate [4, 82, 100, 105, 263, 266], which allows E. coli to produce nearly constant amounts of FtsZ per cell at all growth conditions. A few transcriptional regulators have also been shown to have an effect on transcription initiated at some of these promoters. The stationary phase sigma factor $\sigma^S$ increases transcription from P_{ftsQ1} [17, 263]. A quorum sensing transcription factor SdiA positively regulates transcription from P_{ftsQ2} [300]. RcsB, a regulatory protein from a complex signalling system that activates transcription of genes involved in colanic acid synthesis, upregulates P_{ftsA} [49, 109]. There is also some evidence that the nucleotide ppGpp, the effector of the stringent response, might be a positive regulator of FtsZ expression [291]. The promoters in the ddlB-ftsZ region contribute only about a third of all transcriptional activity entering the ftsZ gene [71, 99]. This means much more work needs to be done before there is a complete understanding of the transcriptional regulation of ftsZ.

![Diagram of the mra cluster with known promoters and RNase E cleavage sites](image)

Fig. 5. Known promoters and RNase E cleavage sites in the mra cluster. T, a transcription terminator. The RNase E cleavage site within the ftsA gene was previously thought to be a promoter (P_{ftsA}) [47].

It has been estimated that there are about 15 000 molecules of the FtsZ protein per average E. coli cell [61, 171, 225]. In contrast, there are only about 200 molecules of FtsA [208, 295] and about 20 molecules of FtsQ [50] per cell. The correct ratio
between FtsZ and FtsA concentrations in a cell is very important for *E. coli* division, as overexpression of either FtsZ or FtsA alone leads to cell filamentation, but overexpression of both of these proteins does not block division [23, 61, 81]. The *ftsQ*, *ftsA*, and *ftsZ* genes are arranged sequentially on the *E. coli* chromosome (Fig. 5), with transcription entering from upstream of the *ftsQ* gene and terminating downstream of the *ftsZ* gene. There are also several promoters located within the *ftsQ* and *ftsA* coding sequences. This results in *ftsZ* having the highest transcriptional activity, followed by *ftsA*, with *ftsQ* having the lowest transcriptional activity of the three genes. However, the final ratio between FtsZ, FtsA, and FtsQ in the cell depends also on post-transcriptional regulation of their expression.

*ftsZ* is translated more efficiently than *ftsA*, which is in turn translated more efficiently than *ftsQ* [208]. Furthermore, expression of these genes is modified by mRNA processing. It was shown that there are at least two RNase E cleavage sites which affect the decay of the *ftsA* and *ftsZ* mRNA. One is within the *ftsA* gene, and another in the *ftsA-ftsZ* intergenic region (Fig. 5) [47]. The RNase E cleavage decreases the relative amount of the *ftsA* mRNA to the *ftsZ* mRNA approximately five-fold [47]. It is not known yet if the mRNA processing is regulated, but it has been reported that RNase E activity can depend on growth conditions [18].

The FtsZ protein is the target of all identified endogenous cell division inhibitors. SulA is induced in response to DNA damage as a part of the SOS response [135, 136]. It inhibits polymerisation and the GTPase activity of FtsZ [142, 286]. In *E. coli* cells, SulA is rapidly degraded by proteases, mainly by Lon, but also by HsIVU [144, 146, 200, 257, 312]. SulA-mediated division inhibition was shown to be fully reversible [178].

SfiC is another FtsZ inhibitor associated with the SOS response [65]. In contrast to SulA, SfiC-mediated division inhibition is irreversible [178]. SfiC is encoded by an excisable 14 kb element e14 that is present in the *E. coli* K-12 chromosome [177]. The element e14 is a defective lambdoid prophage. Excision of e14 has been shown to be induced by the SOS system [42].
A defective prophage Rac was also found to inhibit cell division under conditions of prophage induction. A product of a gene \textit{kil} is responsible for the inhibition which can be reversed by overexpression of FtsZ [57].

MinC, a protein necessary for accurate positioning of the cell division septum (see section 1.1.2 on page 10), is also an inhibitor of FtsZ [140]. MinC requires MinD for activation and correct placement in a cell [67, 70]. A protein of phage origin DicB has been shown to inhibit cell division through the Min system [69]. DicB interacts directly with MinC and activates it to inhibit assembly of the FtsZ ring [140]. The MinC-DicB system inhibits septation at all potential division sites, independently from MinE [69]. DicB is encoded by Kim, another defective lambdoid prophage in \textit{E. coli} K-12 [27, 48].

The prophage Kim was later found to contain one more gene whose product is able to inhibit \textit{E. coli} cell division. The product is an antisense RNA DicF [36]. Its expression is normally completely repressed by DicA, a protein encoded by the same Kim prophage [27]. DicF is complementary to the beginning of the \textit{ftsZ} mRNA. It was shown that DicF can block cell division by inhibiting translation of the \textit{ftsZ} mRNA, possibly by binding to its ribosomal binding site [279]. The division inhibition by DicF is most effective at a high temperature [36]. The DicF RNA was shown to be 53 nucleotides long. Its 3' end is generated by a rho-independent terminator. The 5' end is produced by an RNase III cleavage followed by further processing that requires active RNase E [98]. Analysis of genomes from various bacteria revealed that \textit{dicF} homologues are widespread in prophage-like elements of different origins [97].

Furthermore, the \textit{E. coli} chromosome probably encodes another antisense RNA, StfZ, that can inhibit translation of the \textit{ftsZ} mRNA. It was proposed that StfZ is transcribed from the \textit{ftsA-ftsZ} region of the \textit{E. coli} chromosome, in the direction opposite to that of the genes in the \textit{mra} cluster. It has been observed that cell division is inhibited by high copy numbers of the 2.3 kbp EcoRI fragment that contains \textit{ftsQ}, \textit{ftsA}, and the 5' end of \textit{ftsZ} (Fig. 6) [81, 296]. This effect might be caused by an excess of the FtsA protein, since the correct ratio between the FtsZ and FtsA concentrations is essential for \textit{E. coli} division [61, 81, 296]. However, a shorter 490
bp HindIII-EcoRI fragment, which includes only the 3' end of the \textit{fisA} gene and the 5' end of the \textit{ftsZ} gene (Fig. 6), was also found to block cell division when present in high copy numbers [80]. In addition, the EcoRI fragment was shown to have a strong antisense transcription terminator which would stop transcription from the DNA strand complementary to the one encoding the \textit{ftsQ-ftsZ} genes [243]. Further investigations seemed to indicate that the 148 bp \textit{Bsu36I}-EcoRI fragment (Fig. 6) has a strong antisense promoter [80]. Transcription from such promoter would produce an RNA complementary to the 5' end of the \textit{ftsZ} mRNA, possibly capable of interfering with translation of the \textit{ftsZ} mRNA, in a similar way to the DicF RNA.

![Fig. 6. The \textit{ddIB-ftsZ} region of the \textit{mra} operon. The open reading frames of the four genes are shown as boxes. The scale at the top is given in kilo base pairs. The size of the cloned fragments is given in base pairs. From ref. 80.](image)

A sequence element within the 2.3 kbp \textit{EcoRI} fragment, containing a GC rich inverted repeat preceded by an AT rich sequence, has some similarity with a rho-independent transcription terminator. Assuming that this element acts as the \textit{stfZ} terminator, it was estimated that the StfZ RNA is not more than about 140 nucleotides long [80].

The inhibition of \textit{E. coli} cell division by the cloned \textit{stfZ} gene was observed only when high copy numbers of the gene were present, and only at a high temperature, such as 42°C [80]. It was therefore suggested that the interaction between the antisense StfZ RNA and the \textit{ftsZ} mRNA might require participation of other factors [80].
There is also a possibility that the StfZ RNA is translated, and the translation product inhibits cell division. Sequence analysis revealed that there is one potential open reading frame containing a possible Shine-Dalgarno sequence within the proposed stfZ gene [80]. This open reading frame would code for a 25 amino acid polypeptide.

1.3 Regulatory RNAs

Small untranslated RNAs are found in many different organisms, both prokaryotic and eukaryotic. Such RNAs perform diverse functions, one of which is to regulate gene expression. The mechanisms by which regulatory RNAs accomplish this seem to be very different in eukaryotes and prokaryotes.

1.3.1 In eukaryotes

In eukaryotes, RNAs regulate gene expression through a process called RNA interference (RNAi). It was first observed in transgenic plants as posttranscriptional gene silencing (PTGS), but was later found to exist also in animals and fungi (as quelling). This gene silencing process is conserved in all three kingdoms, since it was shown that induction, target RNA degradation, associated short double-stranded RNAs (dsRNAs), and required proteins are similar in quelling, PTGS, and RNAi [52, 94, 305]. The process is thought to have evolved as a defence mechanism against viruses and transposable elements [305].

The initial signal for the RNAi machinery is a double-stranded RNA. It is rapidly cleaved to produce short RNA pieces, called short interfering RNAs (siRNAs). Any dsRNA segment greater than about 26 bp can be recognised, though longer dsRNAs have been shown to be more effective [222]. Double stranded RNAs can be produced in several ways, including replication of viruses and transcription through repeated copies of transposons. It was suggested that eukaryotic cells might use codon redundancy in mRNAs to avoid long self-complementary regions, since these can trigger RNAi. Alternatively, self-complementarity in mRNAs can be used by cells to make them unstable [305].

The dsRNAs of any origin are then degraded in a two step process. An enzyme with RNase III-like activity cleaves dsRNA in ~22-nucleotide steps, producing ~22 bp
dsRNA fragments with 3' overhangs of 2-3 nucleotides [90]. It is thought that the enzyme stays associated with the double-stranded siRNA, making a small interfering ribonucleoprotein (siRNP) complex. During the next step, siRNP conformation probably changes in such a way that the strands of siRNA become unpaired and can guide the complex to complementary target RNAs [29, 305]. The target RNAs are cleaved in the middle of the recognised sequence, generating small fragments of RNA of the same size as the guiding siRNA, about 22 nt, but with a frame shift of about 11 nucleotides [320].

The RNAi process was found to require proteins other than the RNase III-like ribonuclease. These include helicases, methyltransferases, and even a polymerase, RdRP (RNA-dependent RNA polymerase) [305]. It was recently shown that RdRP can use siRNAs, produced by the initial degradation of the dsRNA, as primers to convert the target RNA into a dsRNA [164, 258]. The dsRNA is then degraded to eliminate the incorporated target RNA while generating new siRNAs for another round of amplification. Such a cycle allows the initial signal for RNAi to be greatly amplified. This could help to explain the observation that even a few trigger dsRNA molecules are sufficient to prevent expression of a target gene over a long period of time. Furthermore, their effect persists through cell division and can spread to other cells [221].

Even though RNAi seems to have evolved mainly as a defence against viruses and transposable elements, evidence is emerging that it is involved in regulation of some endogenous genes as well. It was recently found that RNAs encoded by the cell can regulate gene expression through RNAi. Two Caenorhabditis elegans genes, lin-4 and let-7, encode small temporal RNAs that regulate genes important for stage-specific development of the worm [114]. These two regulatory RNAs are probably members of a large class of small untranslated RNAs found in both invertebrates and vertebrates [153, 154, 155, 159]. There is also data indicating that RNAi might be involved in the regulation of gene expression during plant development. An Arabidopsis gene ago1 is required for RNAi, but mutations in ago1 also cause developmental abnormalities [94]. It is therefore possible that gene regulation by endogenous RNAs through the RNA interference mechanism is more common in
eukaryotes than was previously thought. This hypothesis is also somewhat supported by the finding that an endogenous plant protein rgs-CaM can suppress RNAi [8]. rgs-CaM is a calmodulin-related protein from tobacco plants. Calmodulins normally regulate activities of their target proteins in response to changes in intracellular Ca\(^{2+}\) concentration, thus rgs-CaM might provide a mechanism of RNAi regulation in response to Ca\(^{2+}\) signalling.

In addition to posttranscriptional silencing of genes by degradation of homologous RNA, RNAi machinery may also be involved in methylation of homologous DNA, thus causing transcriptional gene silencing [168, 304].

1.3.2 In prokaryotes

In prokaryotes, most of the regulatory RNAs found so far are expressed by accessory DNA elements such as plasmids, phages, and transposons [260, 294]. Plasmids maintain their copy numbers in bacteria by using negative regulatory systems that correlate the rate of their replication with the number of the plasmids present in the cell at any given time. Most of these systems use an antisense RNA as a negative regulator. Such RNAs are usually encoded from a DNA strand opposite to the one coding for the gene to be regulated, thus they can have perfect complementarity to the target RNA. The rate of plasmid antisense RNA synthesis is typically higher than that of the target RNA [73]. The antisense RNAs are expressed from a constitutive promoter and have a short half life, therefore their concentration in the cells corresponds to the number of plasmids present [73]. There are several methods that antisense RNAs use to inhibit plasmid replication: (1) inhibition of the primer formation, as in ColE1 plasmids [283, 284, 285]; (2) induction of the premature termination (attenuation) of transcription of the essential rep mRNA, as in pT181, pIP501, and pAMβ1 plasmids [40, 41, 156, 217]; (3) inhibition of the Rep protein translation, either by directly blocking the leader peptide translation, as in IncFII plasmids and pLS1 [34, 74, 310], or through inhibition of an activator pseudoknot formation, as in IncB/IncI\(\alpha\) plasmids [12, 308].

In addition to control of copy numbers, plasmid antisense RNAs are also involved in regulation of genes important for plasmid conjugation and plasmid killer systems.
Antisense RNA control is common in bacteriophages as well, where it is mainly used to maintain the lysogenic state [101, 130, 160, 311]. This includes antisense RNA involvement in phage superinfection exclusion systems [232]. One of the systems of antisense control, present in the phages P1, P4, P7, ΦR73, and N15, is notable for the way the antisense RNA is produced. It is cotranscribed with its target RNA, and then processed to generate a pseudo-antisense RNA [56, 101, 236, 247]. Parts of this RNA are complementary to the leader region of the nascent transcript of the lytic operon. Interaction between the pseudo-antisense RNA and the leader region causes premature termination of the target RNA transcription. Transposons have also been shown to express antisense RNAs. A mobile genetic element IS10 encodes an antisense RNA, RNA-OUT, that blocks ribosome binding to an mRNA coding for a transposase Tnp, thus inhibiting its translation [175, 259]. Like in the plasmid copy number control systems, the strength of inhibition by the transposon antisense RNAs is proportional to the number of the transposons present in a cell, hence making such a regulation mechanism very suitable for limiting the numbers of these elements in the cell [294].

Until recently, there were only a few bacterial antisense RNAs found to be encoded by the chromosomal DNA: OxyS, DsrA, MicF, and DicF. An apparent difference from the RNAs encoded by the accessory DNA elements is that the chromosomal antisense RNAs are often encoded by the DNA regions distinct from those coding for the target RNAs, thus the complementarity between the two RNAs is not perfect. Several chromosomal antisense RNAs have also been shown to control multiple genes, which is uncommon in the RNAs encoded by the accessory DNA elements [6].

The OxyS RNA is induced by oxidative stress [7]. OxyS acts as a global regulator that controls expression of many genes, including fhlA and rpoS, which code for transcriptional regulators [5]. The OxyS RNA uses two different mechanisms to control expression of the fhlA and rpoS genes. In the case of fhlA, OxyS binds to the Shine-Dalgarno sequence of the fhlA mRNA, and so blocks ribosome binding and inhibits translation [5]. In the case of rpoS, OxyS represses translation by binding to
the Hfq protein. Hfq is normally required for \textit{rpoS} translation, and OxyS probably alters the Hfq activity [322].

Another chromosomally encoded antisense RNA, DsrA, also controls expression of multiple genes. However, in contrast to the other known bacterial antisense RNAs, it is capable of activation of gene expression, not only repression. DsrA enhances translation of the \textit{rpoS} mRNA. DsrA was shown to bind to the \textit{rpoS} mRNA [179]. This probably alters the secondary structure of the \textit{rpoS} mRNA in a way that allows its increased translation. DsrA also acts as a negative regulator. It binds to the \textit{hns} mRNA, inhibits its translation and enhances its turnover [157, 158]. The H-NS protein is a global transcriptional silencer, thus the action of DsrA activates many genes. The regulation of both \textit{rpoS} and \textit{hns} expression by DsrA requires the Hfq protein [179]. The production of the DsrA RNA is induced by low temperature [264].

The antisense RNA MicF regulates expression of only one gene, \textit{ompF} [199]. \textit{ompF} encodes an outer membrane porin. The MicF RNA binds to the ribosomal binding site of the \textit{ompF} mRNA, inhibits its translation and induces its degradation [9, 75]. The regulation of MicF expression itself is quite complicated. MicF is induced by a variety of conditions, such as high osmolarity, superoxide stress, and temperature changes [229]. Several transcriptional regulators are known to activate MicF expression [75]. The MicF RNA is also destabilised by StpA, an RNA chaperone protein [72].

The DicF RNA is encoded by a defective prophage, and its expression is normally completely repressed [27, 36]. DicF is complementary to the \textit{ftsZ} mRNA. It was shown that DicF can inhibit\textit{ftsZ} translation and block cell division [279].

The majority of prokaryotic regulatory RNAs act through base pairing between the single stranded regions of the regulatory RNAs and the target RNAs. However, some RNAs, such as CsrB [16], control gene expression by interacting with regulatory proteins. A few RNAs, such as OxyS [5], can employ both mechanisms. It was recently found that there are many more small untranslated RNAs encoded by the chromosomal DNA of prokaryotes than was previously thought. Analysis of the \textit{E. coli} genome and a microarray approach helped to identify additional genes encoding
small untranslated RNAs likely to be involved in gene regulation [10, 303]. Several small untranslated RNAs, such as RprA and GcvB [180, 288], have confirmed regulatory functions, but their mechanisms of action are still unknown. It was proposed that the high number of the newly found RNA encoding genes, their diverse expression patterns and high transcription levels indicate that small regulatory RNAs are widespread and play an important role in bacteria [10].

Antisense RNAs expressed in bacteria are usually short, many of them less than 100 nucleotides [294, 302]. RNase processing is often needed to convert the original transcript into an active antisense RNA [98, 101]. Specific bacterial RNases, such as the RNase E, have been shown to affect the half life of the antisense RNAs [162]. Bacterial antisense RNAs usually form one or more stem-loop structures. The loops determine the specificity of interaction between the antisense RNA and the target RNA. Point mutations in the loops have been shown to change the specificity of pairing, while alterations of the loop size strongly affect the binding rates [129, 294]. The loops of the antisense and the target RNAs are involved in the initial interaction, termed the kissing reaction [282]. It has recently been found that many prokaryotic antisense systems have a fairly conserved loop motif YUNR (Y, pyrimidine; R, purine) that possibly supports formation of a structure called the U-turn [11, 103]. The U-turn structures are thought to facilitate rapid interactions between RNAs [102].

The stems of the stem-loop structures in the antisense RNAs have several functions. (1) The stems have to keep the nucleotides involved in the kissing reaction in the single stranded loops. (2) The stability of the stems determines how easily the interaction between the two RNAs will propagate from the initial interaction of the loops to a stable complex [149]. It has been shown that inhibition by some antisense RNAs does not require stable duplex formation between the antisense RNA and the target RNA. In these cases, the kissing reaction between the two RNAs is sufficient, and the inhibition occurs faster than the formation of a stable complex [108]. For other systems, a stable duplex needs to be formed before inhibition can occur [40]. (3) The stem structure is important for the antisense RNA stability. The specific features of the RNA-OUT stem have been shown to make it resistant to both
3'-exoribonucleases and double strand endonucleases [51]. (4) Specific stem structures may also be important for the antisense RNA interaction with the accessory proteins [202].

Some prokaryotic antisense RNAs require accessory proteins for efficient repression of plasmid replication. The ColE1 plasmids express an antisense RNA, RNA I, that interacts with the primer RNA, RNA II, thus interfering with the formation of a stable RNA II-DNA hybrid that is necessary for initiation of plasmid replication. A stable interaction between the antisense RNA I and the primer RNA II is enhanced by the protein Rop (also called Rom) [53, 88, 89]. Deletion of the rop gene has been shown to double the plasmid copy number in slowly growing cells, whilst having almost no effect in fast growing cells [14].
2 Materials and Methods

2.1 Bacterial strains, media, and plasmids

*Escherichia coli* strains used are listed in Table 1. The cells were grown in LB [248], McConkey (Difco), VBA (1 x VB salts [182], 0.2% glycerol, 5 mg/ml casamino acids, 2 μg/ml thiamine), or VBB (1 x VB salts [182], 0.4% sodium succinate, 5 mg/ml casamino acids, 2 μg/ml thiamine) medium. In some cases 1% galactose was added to MacConkey medium. For selection and stabilisation of some strains ampicillin (100 μg/ml), tetracycline (10 μg/ml), chloramphenicol (20 μg/ml), or kanamycin (50 μg/ml) was added to the medium. Unless stated otherwise, bacteria were grown at 37°C.

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<tbody>
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<td>C600</td>
<td>F' leuB6 thr-1 lacA? lacY1 thi-1 supE44 fhuA21 T1R Ph80R Lmb</td>
<td>218</td>
</tr>
<tr>
<td>C600K</td>
<td>F' leuB6 thr-1 galK lacA? lacY1 thi-1 supE44 fhuA21 T1R Ph80R Lmb</td>
<td>laboratory stock</td>
</tr>
<tr>
<td>CSH26</td>
<td>F' ara Δ(lac-pro) thi</td>
<td>274</td>
</tr>
<tr>
<td>DH5α</td>
<td>F' endA1 hsdR17 (rK- mK+) supE44 thi-1 recA1 gyrA (Nal') relA1 Δ(lacZYA-argF) U169 deoR (φ80 dlacΔ(lacZ) M15)</td>
<td>184</td>
</tr>
<tr>
<td>HAT10</td>
<td>F' ara Δ(lac-pro) thi hfq10::cat</td>
<td>274</td>
</tr>
<tr>
<td>IT1528</td>
<td>F' gal-3 Δ(cya) rho-15 ilvY864::Tn10</td>
<td>96</td>
</tr>
<tr>
<td>MG1655</td>
<td>Wild type <em>E. coli</em> K-12 strain</td>
<td>126</td>
</tr>
<tr>
<td>TP8503</td>
<td>F' Δ(lac-proB) leu thi supE42 fhuA T1R Ph80R::Tn7 TmpR</td>
<td>185</td>
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<tr>
<td>W3110</td>
<td>Wild type <em>E. coli</em> K-12 strain</td>
<td>139</td>
</tr>
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</table>

Table 1. *E. coli* strains

The plasmids used for cloning fragments from the *mra* cluster in search of antisense transcription terminators and promoters were pDDC, pddLB, and pZAQ. In plasmid
pDDC, a 5.5 kbp EcoRI-SalI fragment from *E. coli* chromosome that codes for *murD*, *ftsW*, *murG*, and *murC* genes is cloned into vector pUC18 [216]. In plasmid pddlB, a 1.2 kbp *BamHI* fragment coding for *ddlB* gene is cloned into vector pT7-4. In plasmid pZAQ, a 4.4 kbp *PstI-Clal* fragment coding for *ftsQ*, *ftsA*, and *ftsZ* genes is cloned into vector pBR322 [272].

The plasmid pVH1 [124] was used to overexpress LacI protein. Some vectors that were used are shown in Fig. 7. Termination assay vector pJW30 [309] was used to search for transcription terminators. Vectors pKO1 [190], pASHOK (constructed by Ashok Kumar from vector pKK2328 [43]), and pRS551 [261] were used to search for promoters. Translational fusion vector pRS552 [261] was used to make expression constructs containing the ribosomal binding site of *ftsZ* and to subsequently transfer the constructs onto the chromosome of *E. coli*. Vector pKO3 [106, 163] was used for site-specific mutagenesis of the *ftsA*-*ftsZ* intergenic region in an attempt to abolish the function of StfZ.

Table 2 lists some of the plasmids constructed to search for transcription terminators – these plasmids were constructed by straightforward cloning of restriction fragments from the *E. coli mra* region into the vector pJW30. Plasmid pT6 was constructed by digesting plasmid pT1 with *PvuII* and *BamHI*, then blunting the ends and religating. Plasmid pT11 was constructed by digesting plasmid pT2 with *KpnI* and religating so that the 620 bp fragment between two *KpnI* sites in the *ftsQ-ftsA* region was lost. To construct plasmids pTX6, pTX7, and pTX10, a 4803 bp *BamHI-BgII* fragment from plasmid pDDC covering *murD*, *ftsW*, *murG*, and *murC* genes was incompletely digested by *Sau3A*, and the resulting mixture of fragments was cloned into pJW30 cleaved by *BamHI*. PCR-amplification was used to produce the inserts of plasmids pP1, pP2, pP3, pP4, pP6, pP8, pP8A, pP9, pP9A, pP10, pP11, pP13, pT16, pT17, pT18, pT19, pT20, and pT21, because new restriction sites had to be introduced at the ends of the fragments to allow cloning. The plasmid pZAQ was used as a template in all PCR reactions except the one to produce the insert of pP13 – in this case the chromosomal DNA of *E. coli* C600 cells was used as a template. The information about these plasmids is listed in Table 3. The sequences of the primers used in PCR are shown in Table 4.
Fig. 7. Termination assay vector pJW30, promoter assay vectors pKO1, pASHOK, and pRS551, translational fusion vector pRS552, and vector pKO3. P<sub>gal</sub>: Crp-independent P<sub>2</sub> promoter from gal operon; Stops: translation stop codons in all 3 reading frames; ori: pMB1 replication origin; Ter: transcription terminator T1 from E. coli mB operon (vector pRS552 has 4 copies of this terminator); t<sub>r</sub>: transcription terminator from E. coli rpoC gene; repA: temperature sensitive pSC101 replication origin.
### Table 2. Plasmids constructed

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Restriction fragment cloned</th>
<th>Length</th>
<th>Orientation</th>
<th>Cloned into pJW30 sites</th>
</tr>
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<tbody>
<tr>
<td>pT1</td>
<td>BamHI-HindIII from pZAQ</td>
<td>1699 bp</td>
<td>←</td>
<td>BamHI+HindIII</td>
</tr>
<tr>
<td>pT2</td>
<td>BamHI-BglII from pZAQ</td>
<td>971 bp</td>
<td>←</td>
<td>BamHI</td>
</tr>
<tr>
<td>pT2r</td>
<td>BamHI-BglII from pZAQ</td>
<td>971 bp</td>
<td>→</td>
<td>BamHI</td>
</tr>
<tr>
<td>pT3</td>
<td>BglII-HindIII from pZAQ</td>
<td>733 bp</td>
<td>←</td>
<td>BamHI+HindIII</td>
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<tr>
<td>pT8</td>
<td>BamHI-BamHI from pddlB</td>
<td>1150 bp</td>
<td>←</td>
<td>BamHI</td>
</tr>
<tr>
<td>pT8r</td>
<td>BamHI-BamHI from pddlB</td>
<td>1150 bp</td>
<td>→</td>
<td>BamHI</td>
</tr>
</tbody>
</table>

### Table 3. Plasmids constructed

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Primers used in PCR</th>
<th>Insert length</th>
<th>Orientation</th>
<th>Restriction sites used</th>
<th>Vector used</th>
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<tbody>
<tr>
<td>pP1</td>
<td>prim2-1, prim2-2</td>
<td>135 bp</td>
<td>←</td>
<td>BamHI-HindIII</td>
<td>pASHOK</td>
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<tr>
<td>pP2</td>
<td>prim2-1, prim6</td>
<td>154 bp</td>
<td>←</td>
<td>BamHI-HindIII</td>
<td>pASHOK</td>
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<tr>
<td>pP3</td>
<td>prim8, prim9</td>
<td>105 bp</td>
<td>←</td>
<td>EcoRI-BamHI</td>
<td>pRS551</td>
</tr>
<tr>
<td>pP4</td>
<td>prim8, prim13</td>
<td>179 bp</td>
<td>←</td>
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<tr>
<td>pP6</td>
<td>prim8, prim14</td>
<td>362 bp</td>
<td>←</td>
<td>BamHI</td>
<td>pRS551</td>
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<tr>
<td>pP8</td>
<td>prim8, prim19</td>
<td>1259 bp</td>
<td>←</td>
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<td>pRS551</td>
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<tr>
<td>pP8A</td>
<td>prim8, prim19</td>
<td>1259 bp</td>
<td>←</td>
<td>BamHI</td>
<td>pASHOK</td>
</tr>
<tr>
<td>pP9</td>
<td>prim8, prim19</td>
<td>1259 bp</td>
<td>→</td>
<td>BamHI</td>
<td>pRS551</td>
</tr>
<tr>
<td>pP9A</td>
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<td>1259 bp</td>
<td>→</td>
<td>BamHI</td>
<td>pASHOK</td>
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<td>pP10</td>
<td>prim19, prim22</td>
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<td>prim23-1, prim23-2</td>
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<td>BamHI-HindIII</td>
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<td>pASHOK</td>
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<td>HindIII-BamHI</td>
<td>pJW30</td>
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<td>pT17</td>
<td>prim26, prim27-1</td>
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<td>HindIII-BamHI</td>
<td>pJW30</td>
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<td>pJW30</td>
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<td>BamHI</td>
<td>pJW30</td>
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<td>prim6</td>
<td>GTT GAT CCG TAT TTA CCG CGA AGA ATT CAA C</td>
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<td>prim13</td>
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<tr>
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<tr>
<td>prim26</td>
<td>TCA TTG GTA AGC TTC ATT GTT TC</td>
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<td>prim28-2</td>
<td>CTC GTG GAT CCC GGC GTC AAG C</td>
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<tr>
<td>prim29</td>
<td>TGC GTA AGC TTG CAA AGT CCA CGT TC</td>
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The inserts of the plasmids pTX6, pTX7, pTX10, pP1, pP2, pP3, pP4, pP6, pP8, pP8A, pP9, pP9A, pP10, pP11, pP13, pT16, pT17, pT18, pT19, pT20, and pT21 were sequenced.

2.2 RNA isolation

_E. coli_ cells were grown in 10 ml of LB medium up to the optical density (600 nm) of 0.5. The cells were chilled and collected by centrifugation. The total RNA was isolated by a slightly modified Pospiech and Neumann method for preparation of genomic DNA [268]. The cells were resuspended in 500 µl of solution containing 75 mM NaCl, 25 mM ethylenediaminetetraacetic acid (EDTA), and 20 mM Tris-HCl (pH 7.5). Lysozyme to the concentration of 1 mg/ml and approximately 30 U of RNazin (RNase inhibitor, Promega) were added and incubated at 37°C for 30 min. 50 µl of 10% sodium dodecyl sulfate (SDS) and 0.4 mg of proteinase K was added, and the mixture was incubated at 55°C for 2 h, with occasional inversion. 184 µl of 5M NaCl and 736 µl of phenol-chloroform-isooamyl alcohol (25:24:1) was added and incubation was continued at room temperature for 15 min with frequent inversion. The mixture was centrifuged for 15 min, then the aqueous phase was collected and extracted with chloroform. RNA was precipitated with isopropanol, dissolved in H₂O into which RNazin was added, and treated with RQ1 RNase-free DNase (Promega), with subsequent phenol-chloroform extraction and ethanol precipitation. RNA was dissolved in H₂O, and RNazin was added. All solutions, where possible, were treated before use with diethylpyrocarbonate (DEP). The yield of total RNA was approximately 100 µg.

In some cases, in order to increase the amount and quality of isolated RNA, PHASE-Guard™ Heavy (CPG Inc.) was used to facilitate the phenol-chloroform extraction.

To isolate RNA longer than 200 b, Perfect RNA™ Total RNA Isolation Kit, Micro Scale (5 Prime → 3 Prime, Inc.) was occasionally used.
2.3 Southern and Northern blotting

Southern and Northern blots were performed using standard techniques, except for several Northern blotting experiments where to improve the results, NorthernMax-Gly™ (Ambion) kit and protocol was used. DNA or RNA was transferred from a polyacrylamide or agarose gel to a positively charged nylon membrane (Roche) by capillary action or by electrophoresis, and then linked to the membrane by UV light, using a Stratagene Crosslinker. Probes for detecting DNA or RNA were end-labelled with $^{32}$P, using T4 Polynucleotide Kinase and [$\gamma^{32}$P]ATP, and then cleaned with NAP® 5 Columns (Pharmacia Biotech) or with MicroSpin™ G-25 Columns (Pharmacia Biotech). The hybridisation of probes was carried out for 24 to 72 hours, at 35-40°C. After hybridisation the membrane was washed and exposed to x-ray film (Sterling® Diagnostic Imaging) or Storage Phosphor Screen (Molecular Dynamics).

The oligonucleotides that were used as probes in Northern blots (prim4-1, prim4-3, and prim8, Table 5) were designed to be complementary to an RNA that is transcribed from the opposite strand of DNA to that of $ftsA$ and $ftsZ$ genes, at the region of $fisA$-$fisZ$ junction.

2.4 RT-PCR

Reverse transcription coupled PCR (RT-PCR) was performed according to the Pharmacia Biotech protocol, using their Ready-To-Go® RT-PCR Beads. 5-10 μg of total RNA from *E. coli* C600, C600K, and C600K+pT16 strains was used as template. Primers prim4-1, prim31-1, prim31-2, prim31-5, prim31-7, prim31-9, and prim31-10 (Table 5) were designed to be complementary to the 3' end of StfZ RNA (first primer). Primers prim4-2, prim31-3, prim31-4, prim31-6, prim31-8, and prim31-11 (Table 5) were designed to be complementary to the product of reverse transcription (second primer). The oligonucleotide prim4-3 (Table 5) was used as a probe for detection of the RT-PCR product, and it was complementary to the region in between the two primers.
Table 5. Oligonucleotides for Northern blotting and RT-PCR

<table>
<thead>
<tr>
<th>Oligo</th>
<th>as primer</th>
<th>Sequence 5'-3'</th>
</tr>
</thead>
<tbody>
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<td>prim4-1</td>
<td>1&lt;sup&gt;st&lt;/sup&gt;, probe</td>
<td>ATT TTT ATG AGG CCG ACG</td>
</tr>
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<td>prim4-2</td>
<td>2&lt;sup&gt;nd&lt;/sup&gt;</td>
<td>GAT TTG TGC CTG TCG CC</td>
</tr>
<tr>
<td>prim4-3</td>
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</tr>
<tr>
<td>prim8</td>
<td>probe</td>
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</tr>
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</tbody>
</table>

The sequence of RT-PCR was as follows: reverse transcription using the total RNA and the first primer was performed at 42°C for 25 min. Reverse transcriptase was then inactivated at 95°C for 5 min and the second primer added. The 40 cycles of PCR after reverse transcription were performed at the following temperatures: 95°C for 1 min, 45°C for 1 min, and 72°C for 1 min.

To make sure the template RNA was not contaminated with DNA, a negative control was used where reverse transcriptase was inactivated before starting the reactions. For a positive control, primers for amplifying Ser-tRNA were used (SS3 and SS4 for RT-PCR, SS2 for detection of the product, Table 5).
The products of RT-PCR were cleaned by QIAquick PCR Purification Kit (QIAGEN) and then detected by one of two methods. If very small products were expected (e.g. 50 bp), they were electrophoresed in a 10% polyacrylamide gel, then DNA was transferred from the gel to a positively charged nylon membrane (Roche) and hybridised with \( \gamma^{32}P \) labelled oligonucleotide prim4-3 and/or SS2. Hybridisation was performed at 40°C overnight. The membrane was then washed and exposed overnight to an x-ray film (Sterling® Diagnostic Imaging) or Storage Phosphor Screen (Molecular Dynamics). Larger RT-PCR products were electrophoresed in agarose gel and detected using ethidium bromide.

2.5 Primer extension

Primer prim4-1 (Table 5) or prim2-1 (Table 4) was labelled with [\( \gamma^{32}P \)]ATP using polynucleotide kinase. It was annealed to approximately 7 \( \mu \)g of E. coli C600K or MM38 total cellular RNA in 15 \( \mu \)l of reaction mixture containing 10 mM Tris-HCl (pH 8.0), 150 mM KCl, and 1 mM EDTA, by heating the mixture to 94°C for 5 min, and then cooling it slowly to 37°C. 30 \( \mu \)l of solution containing 1.5 x M-MuLV reverse transcriptase buffer, dNTP mix (0.33 mM each), and 20 U of M-MuLV reverse transcriptase (Boehringer Mannheim) was added, and the reaction mixture was incubated at 37°C for 60 min. The products were treated with DNase-free RNase A, extracted with phenol-chloroform, and precipitated with ethanol.

The cDNA products were electrophoresed in the denaturing 10% polyacrylamide gel along with a sequencing ladder. M13mp18 DNA was used as a template for sequencing, which was performed according to the Pharmacia Biotech protocol, using their T7 Sequencing™ Kit and the universal primer. [\( \alpha^{35}S \)]dATP was used for labelling. DNA was transferred from the gel to a nylon membrane and the membrane was exposed overnight to x-ray film (Sterling® Diagnostic Imaging) or Storage Phosphor Screen (Molecular Dynamics).
2.6 Immuno-fluorescent staining

Two methods to prepare *E. coli* cells for immuno-fluorescent staining were used. First method: 100 μl of 16% paraformaldehyde and 1 μl of 25% glutaraldehyde were mixed, then 20 μl of 1 M NaPO₄ (pH 7.2) were added. 500 μl of *E. coli* culture (of OD₆₀₀ at approximately 0.4) were added to the mixture and incubated at room temperature for 10 min, and then on ice for a further 50 min. After that, the cells were washed three times in 500 μl PBS (10 mM NaPO₄ (pH 7.2), 150 mM NaCl, 15 mM KCl), then resuspended in 400 μl GTE (50 mM glucose, 20 mM Tris (pH 7.5), 10 mM EDTA). Such fixed cells were incubated at 4°C for several days, then treated with lysozyme: 40 μl of fixed cells were mixed with 10 μl of 200 to 500 μg/ml lysozyme solution in GTE, left to stand for one minute, and transferred onto a slide pre-coated with 0.1 % poly-L-lysine. After three minutes at room temperature the excess liquid was removed, the cells on the slide were washed two times in PBS, and the slide was allowed to air-dry completely (about 30 min).

The second method of preparing cells takes much less time and seems to work just as well. 10 μl of *E. coli* culture were transferred onto a slide pre-coated with 0.1 % poly-L-lysine and left at room temperature for 5-10 minutes. Excess liquid was removed and the cells on the slide were fixed by adding 10 μl of methanol:acetic acid:water (3:1:4) solution and incubating for 10 minutes at room temperature. The cells were then washed three times with PBS and permeabilised by adding 10 μl of 0.4 to 8 mg/ml lysozyme solution in GTE and incubating for 10 min at room temperature. Finally, the cells were washed three times in PBS.

The prepared cells were treated with antibodies in the following way. 10 μl of PBS were added to the cells on slide, left there for five minutes and removed. 10 μl of 2% BSA solution in PBS were added, left for 15 min at room temperature and removed. 10 μl of polyclonal anti-FtsZ antibody (1/500 dilution in 2% BSA/PBS) were added, left overnight at 4°C (the slide was kept during this incubation period inside a petri dish wrapped in parafilm, together with a piece of wet paper – this was done to prevent drying out of the antibody solution), and then washed ten times with PBS. 10 μl of CY3 anti-mouse secondary antibody (1/250 dilution in 2% BSA/PBS)
were added, left for 1 hour at room temperature in the dark (with a piece of wet paper in petri dish, wrapped in parafilm), and then washed eight times with PBS. 10 μl of 10% glycerol solution in PBS were added and left for 5 min. In some cases, 10 μl of 200 ng/ml DAPI (4,6-Diamidino-2-phenylindole) solution in PBS were added instead of PBS alone since DAPI binds DNA and makes it visible under UV light. PBS (or DAPI/PBS) was removed, and 5 μl of SlowFade Antifade reagent in glycerol/PBS (Molecular Probes) were added. The cells were then covered with a coverslip and could be looked at under the microscope.

2.7 β-galactosidase assay

In experiments with *E. coli* TP8503 [λR2], TP8503 [λR5], and TP8503 [λR7], cells for β-galactosidase assays were taken from one of three types of culture:

- an overnight culture, or
- a culture that was diluted 100 times from an overnight culture and grown up to OD₆₀₀ of about 0.3, or
- a culture that was diluted 100 times from an overnight culture and grown up to OD₆₀₀ of about 0.3, then diluted and grown up to OD₆₀₀ of 0.3 for three more times to get rid of any effects that stationary phase could have on the expression of β-galactosidase. This was done to see if expression of β-galactosidase is dependent on the growth phase of the culture.

β-galactosidase activity was determined by the method described by Miller [195]: The OD₆₀₀ of the desired culture was measured. 0.5 ml of the culture was mixed with 0.5 ml of Z buffer (when β-galactosidase activity was expected to be high, 0.1 ml of culture was mixed with 0.9 ml of Z buffer). As a control, 0.5 ml of LB medium was mixed with 0.5 ml of Z buffer. 50 μl of chloroform was added and the mixture was vortexed for 15 seconds. The sample was stored at 4°C until ready to assay. Next, 200 μl of 4 mg/ml o-nitrophenyl-β-D-galactopyranoside (ONPG) were added, the sample was transferred to a 30°C water bath and incubated until yellowing occurred. To stop the reaction, 0.5 ml of 1 M Na₂CO₃ was added and the sample was transferred to ice. OD₄₂₀ and OD₅₅₀ of the sample were measured and the β-
galactosidase activity was calculated and expressed in Miller Units using the equation:

\[
\frac{OD_{420} - 1.75 \times OD_{550}}{OD_{600} \times V \times T} \times 1000
\]

Where \( V \) is the culture volume (in millilitres) taken for assay,

\( T \) is the duration (in minutes) of sample incubation at 30°C.

Z buffer:

<table>
<thead>
<tr>
<th>Compound</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na(_2)HPO(_4)</td>
<td>4.26 g</td>
</tr>
<tr>
<td>NaH(_2)PO(_4) \cdot H(_2)O</td>
<td>3.11 g</td>
</tr>
<tr>
<td>KCl</td>
<td>0.375 g</td>
</tr>
<tr>
<td>MgSO(_4) \cdot 7H(_2)O</td>
<td>0.123 g</td>
</tr>
<tr>
<td>(\beta)-mercaptoethanol</td>
<td>1.35 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.25 ml</td>
</tr>
<tr>
<td>H(_2)O</td>
<td>to 500 ml</td>
</tr>
</tbody>
</table>

2.8 Western blotting

For Western blots, crude cell extracts were used. The crude extracts were prepared in the following way: *E. coli* cells were grown to the desired optical density, one or more millilitres of culture were taken and centrifuged for 1 min. The cells were then washed twice in 1 ml of ice-cold 50 mM Tris-HCl (pH 7.4) and resuspended in 100 μl of H\(_2\)O. 100 μl of 2 x cracking buffer (50 mM Tris-HCl, pH 6.8; 100 mM dithiothreitol, 2% SDS, 0.1% bromphenol blue, and 10% glycerol) was added, the tube with cells in the cracking buffer was placed into boiling water bath for 5 min and then centrifuged for 1 min. The resulting supernatant was used as crude cell extract.

The concentration of total protein in crude cell extracts was measured by dotMETRIC™ 1μl Protein Assay (Chemicon). To separate proteins, the extracts were run on 13 % SDS-PAGE gels. Two identical gels were run at the same time—one for coomassie staining to see how much of the total protein is in each lane, and the other for Western blotting. Western blotting was performed using standard techniques. ECL Western Blotting System (Amersham Biosciences) was used for detection of proteins.
2.9 Other methods
DNA was sequenced according to the Perkin Elmer protocol, using their dRhodamine Terminator Cycle Sequencing Ready Reaction Kit. *E. coli* cells were transformed with plasmid DNA either by the CaCl/RbCl method or by electroporation [248]. Plasmid DNA was isolated by the alkaline lysis method or by using Wizard® Plus SV Minipreps Kit (Promega). DNA fragments before ligations were cleaned by Wizard® DNA Clean-Up System (Promega) or by QIAquick Gel Extraction Kit (Qiagen). The enzymes for DNA manipulation were those from Fermentas, Promega, and New England Biolabs. The standard DNA methods were performed according to ref. 248.
3 Antisense transcription in mra region of E. coli chromosome

Antisense RNAs regulate the expression of several bacterial chromosomal genes. Such regulation can be quite effective, being both energy efficient and quick. This energy efficiency is achieved in two ways. Firstly, to produce a regulator a cell needs only to make an RNA, and the protein synthesis step is omitted. Secondly, unlike with inhibition of proteins, antisense RNA interferes with the translation of the target RNA, so no protein gets produced. Target RNAs are produced continuously (if there is no other regulation), so protein production can resume immediately after antisense RNAs disappear. Consequently, the antisense inhibition or activation can be stopped more rapidly than the transcriptional one, especially if the antisense RNA involved has a short half life.

Currently only four chromosomally encoded antisense RNAs are known to control gene expression in E. coli [302]. Why so few, if antisense regulation appears to be so effective? Recently, analysis of the E. coli genome and a microarray-based search have identified many new small untranslated RNAs encoded by the chromosomal DNA that might be involved in gene regulation [10, 303]. At the start of this project, however, such information was not available. With this in mind, our aim was to check if regulation by antisense RNAs is more widespread in bacteria than it appeared at the time.

To start with, we decided to search for any genes in the mra region of the E. coli chromosome that are being transcribed in the opposite orientation to the genes mraZ..envA. Such transcripts would very likely be able to act as antisense RNAs. The mra region was selected for several reasons. There is a possible antisense gene stfZ located at the ftsA-ftsZ intergenic region in the mra gene cluster [80]. Also, the mra gene cluster contains many genes essential for cell division and cell wall production. Their regulation is important and might need to have several levels of control to let the cell coordinate the complex events of cell division.

In search for antisense genes in the mra region, two methods were used. The first was to look for antisense promoters – a promoter would mean that there is something
being transcribed in the antisense orientation. The second was to look for antisense transcription terminators, since terminators are also usually associated with genes.

### 3.1 Promoters

To search for antisense promoters, a DNA segment containing *E. coli* genes *ftsQ*, *ftsA*, and *ftsZ* was fragmented into small pieces which were then cloned into promoter assay vectors to test for promoter activity. The 4372 bp *ftsQ-ftsZ* DNA segment was obtained from the plasmid pZAQ by digesting it with endonucleases *PstI* and *ClaI*. After that, it was digested incompletely by endonucleases *Alul*, *Bsh1236I*, *HaeIII*, and *RsaI* to get blunt-ended DNA fragments of about 200 bp in length. These were ligated into the *Smal* site of promoter assay vector pKO1 or pASHOK (Fig. 7, p. 36).

The vector pKO1 has *galK* as a reporter gene. Therefore pKO1 constructs were transformed into *E. coli* C600K (*galK*) cells. These were grown on MacConkey plates containing galactose to test for promoter activity of the inserted DNA fragments. On such plates colonies producing galactokinase (promoter⁺) are red, while colonies that do not produce galactokinase (promoter⁻) are white.

The vector pASHOK has *lacZ* as a reporter gene. pASHOK constructs were transformed into *E. coli* TP8503 (*lacZ*) cells that were then grown on LB plates containing X-gal. On such plates colonies producing β-galactosidase (promoter⁺) are blue, while colonies that do not produce β-galactosidase (promoter⁻) are white.

None of the colonies of the *E. coli* cells carrying the constructed plasmids had the colour indicating promoter activity in the cloned DNA fragment. This could have happened for several reasons. The blunt-end ligation used in the experiment was not very efficient, thus the constructs obtained most probably did not include all the possible DNA pieces that could be made by *Alul*, *Bsh1236I*, *HaeIII*, and *RsaI* digestion of the *ftsQ-ftsZ* DNA fragment. Also, using specific enzymes for digestion limits the diversity of the fragments, so a potential promoter site could have been cut into two, or a terminator could have been included at the end of the fragment. In either case, this would mask the promoter activity. The method used was clearly not
good enough for finding promoters since it failed to detect even the sense promoters that are located inside the \textit{ftsQ} and \textit{ftsA} genes.

### 3.2 Terminators

To search for terminators, a termination assay vector pJW30 (Fig. 7, p. 36) was used. The fragments tested are shown in Fig. 8. They were ligated into pJW30 between \textit{P}_{gal} promoter and \textit{gaK} gene. The transcription termination activity was assayed by the colour of \textit{E. coli} C600K (\textit{galK}) cells that carried the constructs while the cells were growing on MacConkey plates containing galactose. On such plates colonies producing galactokinase (terminator\textsuperscript{+}) are red, while colonies that do not produce galactokinase (terminator\textsuperscript{-}) are white.

In all the constructs tested, every insert that was transcribed in the opposite orientation to that of the genes in the \textit{mra} cluster (antisense orientation) has shown strong transcription termination activity. On the other hand, none of the inserts that were transcribed in the same orientation as that of the genes in the \textit{mra} cluster have shown any termination activity (Fig. 8). The fragments cloned in the sense orientation were used as a control, since the only known terminator for the genes in the \textit{mra} cluster is downstream of the \textit{envA} gene [21]. One possible explanation for the presence of terminators in all the antisense fragments tested is that these are rho-dependent transcription terminators that require that RNA being transcribed is not translated at the same time.

The sequences that specify a rho-dependent terminator are very diverse and do not have a simple consensus. It is known that Rho protein has high affinity for single stranded RNA with some C residues, but no obvious clustering or regular spacing is necessary [240]. Termination occurs when the Rho protein catches up with an RNA polymerase at the elements that force the RNA polymerase to pause – the result is that RNA is released [240]. Because there is no stringent sequence requirement, rho-dependent terminators might be quite frequent in DNA sequences. In \textit{E. coli}, rho-dependent terminators are common within genes as well as at the ends of genes and operons [239], but the ones within genes usually function only when nascent RNA is not being translated by a ribosome [241, 246]. The Rho protein has to bind RNA to terminate transcription, but it would not be able to gain access if the RNA is blocked.
by a ribosome that is in the process of translating the RNA. It has also been observed that in vitro in the presence of Rho protein, E. coli lacZ fragments have stronger termination activity when transcribed in the antisense orientation than the same fragments in the normal expressed orientation [John P. Richardson, personal communications].

To rule out rho-dependent termination, two strategies could be employed. The first would be to use a rho' strain of E. coli. Unfortunately, rho mutants display many other defects in addition to suppression of transcription termination [95] which makes them difficult to use. The other strategy would be to clone only very short fragments of DNA, since the sequences necessary for rho-dependent termination extend over 150-200 bp [240].

To achieve this, a 4803 bp fragment from the mra region containing 'murD, ftsW, murG, and murC' was incompletely digested by Sau3A, an endonuclease with a 4 bp recognition sequence, and the resulting fragments were cloned into pJW30. E. coli C600K transformants with no galactokinase activity were selected, so that the plasmids they carried would have inserts with transcription termination activity. When the approximate size of the plasmids was determined, it became apparent that most of the inserts were much longer than 200 bp. The smallest three plasmids (pTX6, pTX7, and pTX10, Fig. 8) were selected and their inserts sequenced. Size of the inserts ranged from 357 to 601 bp, not small enough to exclude rho-dependent termination.

So, no fragments of DNA that would both have termination activity and also be short enough to exclude rho-dependent termination were found in the murD-murC region. Since only one endonuclease, Sau3A, was used to produce the DNA fragments, it could have cut through a termination site thus inactivating it. Furthermore, Sau3A has only 19 recognition sites in the murD-murC region, so resulting fragments were not varied enough to contain a sufficient amount of DNA fragments of less than 200 bp to cover all parts of the murD-murC region. To check if there are rho-independent terminators in the antisense strand of the mra region, more experiments would be needed.
Fig. 8. A search for transcription terminators. Fragments were cloned in the indicated direction into HindIII and/or BamHI sites of transcription termination assay vector pJW30 (Fig. 7, p. 36), between promoter P_{mur} and the galK gene. The presence of galactokinase activity was determined by the colour of *E. coli* C600 (galK) cells carrying the constructs when grown on MacConkey plates containing galactose. B, BgIII; BH, BamHI; B36, Bsu36I; ER, EcoRI; H, HindIII; K, KpnI; P, PvuII.
It is possible, though, that all the termination activity observed in the experiments is rho-dependent. It could be that cells are using rho-dependent termination mainly to stop unnecessary or even harmful transcription (in case a functional antisense RNA forms) of the strand of DNA opposite to that of normal genes. More investigation would be needed to confirm this: first to prove that the observed termination was rho-dependent, and then to see if such termination in the antisense direction is common throughout the chromosome.

However, the original purpose of the experiments was to find if there are genes that are transcribed from the antisense strand of the mra region. For that, discriminating between rho-dependent and rho-independent terminators might be unnecessary. Antisense RNAs are not translated, thus ribosomes would not interfere with binding of Rho protein and so both rho-dependent and rho-independent termination could be used to produce them. Unfortunately, there is no easy way to find if the terminators detected in the experiments are used for antisense genes or just to stop unnecessary random transcription. It means a different method is needed to look for genes encoding antisense RNAs.
4 Antisense RNA StfZ

Dewar and Donachie have previously reported that a 490 bp DNA fragment that extends over the ftsA-ftsZ gene junction in the mra cluster of the E. coli chromosome can inhibit cell division [80]. There was evidence that the fragment contains an antisense promoter and terminator. It was therefore proposed that the fragment contains an antisense gene stfZ [80]. An RNA produced from such a gene might be able to work as an antisense RNA, which could then be responsible for the inhibition of cell division. This chapter is going to demonstrate that the StfZ RNA is indeed produced in E. coli cells.

4.1 The proof of StfZ existence

Several methods have been used to detect StfZ RNA and to show that it is normally present in E. coli cells. The Northern blot experiments allow direct detection of a specific RNA. Unfortunately, it proved to be extremely difficult to detect StfZ RNA using Northern blots. The alternative method, reverse transcription coupled PCR (RT-PCR), relies on amplification of a target RNA before detection, making it possible to detect even very small amounts of RNA. However, it is a less preferable method to Northern blot, because it does not enable one to determine the exact length of the target RNA, and also the amplification step of RT-PCR can lead to false positive results.

4.1.1 Northern blot

Ten Northern blot experiments using total RNA isolated from E. coli strains MG1655, C600, C600K, MM38, MM38 rne-1, MM38 rncA, and MM38 pnp7 rncA were carried out, and they were all unsuccessful – that is, no StfZ RNA could be detected. Total RNA was isolated from cells grown at different temperatures (30°C, 37°C, or 42°C), in case StfZ RNA was produced only at a certain temperature range. RNase E and RNase III are both involved in degradation of RNAs in E. coli cells, therefore MM38 rne-1, MM38 rncA, and MM38 pnp7 rncA strains were used to improve the chances of detecting StfZ RNA. The MM38 rne-1 strain has a mutation in the gene coding for RNase E, making it temperature-sensitive. The gene for
RNase III is deleted in the MM38 \( rnc\Delta \) strain. This deletion causes an increase in PNPase concentration in cells [276, 277]. To see if the effect of \( rnc\Delta \) (if any) is due to the absence of RNase III, and not to the increase of PNPase, a strain with a null mutation in \( pnp \) was used, namely MM38 \( pnp7 \ rnc\Delta \). StfZ RNA could not be detected in any of these experiments.

In the following Northern blot experiment, commercially available RNA was used – Total RNA from \( E. \ coli \) (Ambion). 10 \( \mu \)g and 20 \( \mu \)g of Ambion RNA were run in a 5\% polyacrylamide gel together with Century-Plus RNA markers (Ambion). For Northern blotting, NorthernMax-Gly\textsuperscript{TM} (Ambion) reagents and protocol were used. \( \gamma\textsuperscript{-}^{32}\text{P} \) labelled probes prim4-1, prim4-3 and prim8 (Table 5, p. 41) were used for detection of StfZ RNA. Hybridisation was performed for three days at 37\(^{\circ}\)C with all three probes simultaneously. Then the nylon membrane with bound RNA was exposed to a Storage Phosphor Screen (Molecular Dynamics) for three days. The results are shown in Fig. 9. The bands corresponding to StfZ RNA are very faint. This confirms that the amount of StfZ RNA in cells is quite small. Unfortunately, the bands of Century-Plus RNA markers are not visible, therefore the length of StfZ RNA cannot be determined. To see the RNA markers, the polyacrylamide gel has been made with ethidium bromide, but the markers were not visible under available UV light. In a separate experiment, the RNA markers were end-labelled with \( ^{32}\text{P} \), but this treatment degraded the RNA to the point where separate bands of RNA could not be reliably distinguished.

![Northern blot](image)

**Fig. 9.** A northern blot. 20 \( \mu \)g (lane 1) and 10 \( \mu \)g (lane 2) of Ambion RNA have been run in a 5\% polyacrylamide gel. StfZ RNA was detected by \( \gamma\textsuperscript{-}^{32}\text{P} \) labelled probes prim4-1, prim4-3 and prim8 (Table 5, p. 41).
4.1.2 RT-PCR

To confirm the results of the only successful Northern blotting experiment, reverse transcription coupled PCR was used, as it amplifies the signal and much smaller amounts of RNA can be detected. To make sure the template RNA was not contaminated with DNA (as even small amounts of DNA could cause false positive results), a negative control was used where reverse transcription before PCR reactions was omitted. To avoid detecting unspecific products, a probe was designed to be complementary to the sequence between the two primers used in the PCR (Fig. 10).

![Diagram of RT-PCR process]

Fig. 10. The steps of RT-PCR. Total RNA from *E. coli* cells is mixed with a primer that is complementary to the StfZ RNA. Reverse transcriptase extends the primer to produce a DNA strand complementary to StfZ. After this reaction, reverse transcriptase is inactivated, a second primer is added, and PCR cycles that use thermostable DNA polymerase are started. The products of the PCR reaction are run in a polyacrylamide gel, then transferred to a nylon membrane, and hybridised to a labelled probe that is complementary to StfZ.

The results of one of the RT-PCR experiments are shown in Fig. 11. Primers prim4-1 and prim4-2 (Table 5, p. 41) were used to amplify StfZ RNA, and an oligonucleotide prim4-3 (Table 5, p. 41) was used to detect the product. The resulting band corresponds to a product about 50 bases long, as expected. Other RT-PCR experiments have been carried out with different primers, as shown in section 4.2.2, and they also confirm that StfZ RNA is produced in normal *E. coli* cells.

The existence of StfZ RNA is also verified by the primer extension experiments that have been carried out to find the 5' end of StfZ RNA (section 4.2.1).
Fig. 11. RT-PCR experiment. 1, positive control (amplified Ser-tRNA, primers used: SS3 and SS4 (Table 5, p. 41)); 2, negative control (same as 1 but reverse transcriptase (RT) is inactivated); 3, negative control (same as 4 but RT is inactivated); 4, StfZ RT-PCR (primers used: prim4-1 and prim4-2 (Table 5, p. 41)); 5, $^{32}$P-labelled oligonucleotide (46 bases). Total RNA used was purified from E. coli C600K cells that had been grown to log phase in LB medium at 37°C. RT-PCR products were detected by Southern blot. $^{32}$P-labelled oligonucleotide prim4-3 (Table 5, p. 41) was used to detect StfZ, $^{32}$P-labelled oligonucleotide SS2 (Table 5, p. 41) was used to detect Ser-tRNA.

4.2 Finding the length of StfZ

4.2.1 Primer extension

Since Northern blotting experiments had not worked well, some other method was needed to find the length of the stfZ gene. Primer extension allows one to determine the 5' end of an RNA. In such experiments, total RNA extracted from cells is hybridised to a labelled oligonucleotide that is complementary to the RNA so that it acts as a primer for reverse transcription (Fig. 12). The length of the product of the primer extension can be found out, and so the 5' end of the RNA determined. In practice, though, reverse transcription using M-MuLV reverse transcriptase is performed at quite a low temperature, 37°C, and at such a temperature the target RNA can be folded into a secondary structure that would interfere or even prevent reverse transcription, so leading to identification of incomplete products.
Fig. 12. The steps of primer extension. A primer complementary to the StfZ RNA is labelled with $^{32}$P and then annealed to the total RNA from *E. coli* cells. Reverse transcriptase extends the primer to produce a DNA strand complementary to StfZ. This product is run in an acrylamide gel together with DNA sequencing reactions that help determine the length of the product.

Primer extension experiments using primer prim4-1 (Table 5, p. 41) and the total RNA of *E. coli* C600K have yielded several products (Fig. 13). The two most abundant products are 44 bases and 93 bases long, corresponding to the stops of reverse transcription shown in Fig. 14. However, as RT-PCR experiments discussed in the following section indicate, the stop in the 93 b product cannot be the true 5' end of the StfZ RNA. It is most likely that this stop in reverse transcription is induced by a secondary structure, as almost certainly is also in the case of the shorter 44 b product. There are also several very faint bands of longer products visible, but because of their weakness it is difficult to say if any of them is the true product indicating the 5' end of the StfZ RNA – they may be other intermediate products, or perhaps even unspecific products. M-MuLV reverse transcriptase is not good at extending long sequences, so if the 5' end of the StfZ RNA is very far from the annealing place of the primer prim4-1, most of the products could be incomplete. Primer extension experiments with prim4-1 have also been performed using *E. coli* MM38 total RNA, with the same results (not shown here).
A primer extension experiment. A, C, G, and T are sequencing reactions used here as size standards. For sequencing, M13mp18 DNA was used as a template and universal primer as a primer. For primer extension, total RNA from *E. coli* C600K was used as a template and prim4-1 (Table 5, p. 42) as a primer.

**Fig. 13.** A primer extension experiment. A, C, G, and T are sequencing reactions used here as size standards. For sequencing, M13mp18 DNA was used as a template and universal primer as a primer. For primer extension, total RNA from *E. coli* C600K was used as a template and prim4-1 (Table 5, p. 42) as a primer.

**Fig. 14.** The stops of reverse transcription in primer extension experiments. The stops are indicated by black triangles (in experiments with the primer prim4-1) or an open triangle (in an experiment with the primer prim2-1). The dotted arrows indicate an 8 bp inverted repeat that is probably important in secondary structure formation of both StfZ RNA and ftsZ mRNA.

5'...GTTGGC TCGTGGATCA AGCGACTCAA TAGTTGCTG CGAAAAGAGT TTTATTTTT
prim4-1
r-ftsZ

ATGAGGCAGA CGATGATTAC GGCCTCAGGC GACAGGCACA AATCGGAGAG AAACATGTT
Bsa36I

TGAACCAATG GAACTTACCA ATGACGCGGT GATTAAAGTC ATCGGCGTCG GCGGCGGCGG

CGGTAATGCT GGTGAACACA TGGTGCGCGA GGCATTGAA GGTGTGAAAT TCTTC...3'
EcoRI

58
An oligonucleotide prim2-1 (Table 4, p. 38) has also been tried as a primer for the primer extension experiments. In this case, total RNA from *E. coli* MM38, MG1655, or C600K was used as a template. A product of about 46 bases was detected when total RNA of MG1655 and C600K was used (Fig. 15). It corresponds to a transcription stop shown in Fig. 14. In the antisense strand of DNA, the stop is downstream from one of the transcription stops determined by primer extension with prim4-1 (Fig. 14), and therefore it is probably an abortive stop caused by the secondary structure of the StfZ RNA.

![Fig. 15. Primer extension experiments using prim2-1 (Table 4, p. 38) as a primer. Lane 1: with C600K total RNA as a template. Lane 2: with MG1655 total RNA as a template. G, A, T, C are sequencing reactions used here as size standards. For sequencing, M13mp18 DNA was used as a template and universal primer as a primer.](image)

The potential 5' ends of StfZ that are indicated by the products of the primer extension reactions are all inside the limits of the StfZ RNA that have been later found by RT-PCR experiments (described in the section 4.2.2 below). One product, 93 bases long when transcribed from prim4-1, is quite abundant. It would be interesting to find out if StfZ is processed after its production and so there are several
different length StfZ RNAs. RT-PCR is a more sensitive method than primer extension, so maybe it can detect a precursor StfZ RNA that primer extension does not.

4.2.2 RT-PCR
Several reverse transcription coupled PCR experiments have been carried out to see if they could show the approximate length of StfZ RNA. The assumption is that if an RT-PCR experiment is successful and there is a product, then the primers that have been used in the experiment are covering StfZ RNA. For negative results, even though most of the reactions have been repeated to confirm them, one cannot give a conclusive explanation. It would seem then that one or both of the primers used is external to StfZ RNA, but there are problems with that explanation, the same ones that apply to primer extension experiments. Reverse transcription, the most important step in RT-PCR, is sensitive to secondary structures formed by RNA. This reaction has been performed at 42°C, so secondary structures could be present in StfZ RNA and prevent reverse transcription across some sections of the RNA. Also, reverse transcriptases are usually not very good at extending long sequences, thus long products could fail to be produced.

The RT-PCR experiments that have been performed are shown in Fig. 16. A solid red line indicates the extension of StfZ RNA that is confirmed by the experiments. A dashed red line indicates the region between the last successful primer and the first unsuccessful primer. As negative results in RT-PCR are inconclusive, some other method would be needed to find out the exact extension of StfZ RNA.
Fig. 16. RT-PCR experiments performed to find out the length of StfZ RNA. The sequences of primers used are shown in Table 5, p. 41. The black lines represent the products of successful RT-PCR reactions, the grey lines represent the expected products of unsuccessful RT-PCR reactions.
4.3 Search for \textit{stfZ} promoter

To look for an \textit{stfZ} promoter, some DNA fragments from upstream of the possible location of the \textit{stfZ} gene have been cloned into promoter assay vectors pASHOK and pRS551 (Fig. 7, p. 36). The fragments are shown in Fig. 17. They were cloned into the \textit{BamHI} or \textit{HindIII-BamHI} sites of pASHOK; or else into the \textit{BamHI} or \textit{BamHI-EcoRI} sites of pRS551, in front of the \textit{lacZ} gene (Table 3, p. 37). The resulting plasmids were transformed into \textit{E. coli} TP8503 (Δlac) cells. Their β-galactosidase activity was used as an indicator of promoter activity of the cloned fragments. In most cases, the cells carrying the plasmids were grown at 37°C in liquid LB medium overnight, then their β-galactosidase activity was determined by a method described by Miller [195]. In case of the plasmids pP8A, pP9A, and pP10, the presence of β-galactosidase activity was determined by the colour of cells carrying the plasmids when grown on LB plates containing X-gal.

Most of the fragments have been cloned into the vectors in such an orientation that they would show promoter activity for the \textit{stfZ} gene (antisense orientation). None of them were found to have any significant promoter activity (Fig. 17, Table 6). Specific β-galactosidase activity of cells with the plasmids pP3, pP11 and pP13 is slightly higher than the base activity of the vectors used, but the difference is too small for the cloned fragments to be counted as promoters. The fragments of the plasmids pP9 and pP9A have been cloned in the sense orientation and have been used as positive controls, showing that promoter activity can be detected in the systems used. Both pP9 and pP9A contain a promoter for \textit{envA}, therefore β-galactosidase activity of cells carrying these plasmids is high.

To test if the cloned fragments of DNA have higher promoter activity when the cells carrying them are grown at temperatures other than 37°C, these cells were incubated on LB + X-gal plates at 30°C, 37°C, and 42°C. No differences in the colours of the colonies were observed, thus promoter activity does not vary significantly at these temperatures.
Fig. 17. A search for stfZ promoters. The fragments shown here have been cloned in the indicated direction in front of the lacZ gene in promoter assay vectors pASHOK and pRS551 (Fig. 7, p. 36). Inserts of the plasmids pP1, pP2, pP8A, pP9A, pP10, pP11, and pP13 have been cloned into the vector pASHOK, and inserts of the plasmids pP3, pP4, pP6, pP8, and pP9 have been cloned into vector pRS551 (Table 3, p. 37). To determine β-galactosidase activity, the plasmids have been transformed into E. coli TP8503 cells. The β-galactosidase activity of cells with pP1, pP2, pP3, pP4, pP6, pP8, pP9, pP11, and pP13 was measured by a β-galactosidase assay method described by Miller [195]. The presence of β-galactosidase activity in pP8A, pP9A, and pP10 was determined by the colour of the cells carrying the plasmids when grown on LB plates containing X-gal.
The preceding experiments have shown that StfZ RNA is produced, so there must be a promoter for the \( stfZ \) gene. Why then do none of the cloned fragments show promoter activity? The constructs cover more than a kilobase of sequence in the antisense strand of \( E. coli \) chromosomal DNA, upstream of the possible location of the \( stfZ \) gene, as determined by RT-PCR experiments (section 4.2.2). It seems unlikely that the \( stfZ \) promoter could be located even further upstream.

There is a possibility that the level of supercoiling in plasmids carrying the DNA fragments tested for promoter activity is different from that in the chromosome. This would be important if the promoter of \( stfZ \) is sensitive to supercoiling. It has been shown that DNA supercoiling affects gene expression in bacteria [86]. Extracellular osmolarity influences DNA supercoiling in cells [127, 137], therefore, to test this theory, \( E. coli \) TP8503 cells carrying plasmids pP1 - pP13 were incubated on LB + X-gal plates with varying salt levels (without NaCl, 10 g/l NaCl, 20 g/l NaCl, or 30 g/l NaCl).
g/l NaCl), at 30°C, 37°C, or 42°C. No significant differences in the colours of the colonies were observed, thus it is unlikely that the different level of plasmid supercoiling was the reason why no promoter activity was found in the constructs tested.

Another possibility is that the stfZ promoter could not be detected because in the experiments described above β-galactosidase activity was used as an indicator. For β-galactosidase activity to show up, translation of the lacZ transcript is required. In contrast, the StfZ RNA does not require translation for its action. In the plasmid constructs, the 5’ end of the StfZ RNA is likely to be cloned together with the stfZ promoter. There is a possibility that it could form a secondary structure that would include the 5’ end of the lacZ mRNA and would inhibit translation of the lacZ mRNA by hiding the ribosomal binding site of lacZ. To resolve this, one would need to look for the lacZ mRNA transcripts instead of the product, β-galactosidase.

4.4 Search for stfZ terminator

To look for an stfZ terminator, some DNA fragments have been cloned into termination assay vector pJW30 (Fig. 7, p. 36), in front of the galK gene at the HindIII-BamHI or BamHI sites. The fragments are shown in Fig. 18. The transcription termination activity was assayed by the colour of E. coli C600K (galK) cells carrying the constructed plasmids while the cells were growing on MacConkey plates containing galactose. On such plates colonies producing galactokinase (terminator-) are red while colonies that do not produce galactokinase (terminator+) are white.

The insert of the plasmid pT16 is almost identical to that of pSD56, a plasmid constructed by Susan Dewar to show the presence of a terminator in the region [80]. Both these inserts demonstrate termination activity. They are both more than 450 bp long, therefore transcripts could be terminated by either a rho-independent or a rho-dependent mechanism. The sequence of the inserts contains an 8 bp inverted repeat (Fig. 14, p. 58). It has been hypothesized that this inverted repeat is a part of the antisense terminator for the stfZ gene [80]. The plasmids pT17, pT18, and pT19 were constructed to include the inverted repeat while keeping their inserts short enough to
exclude the possibility of rho-dependent termination. None of them show any termination activity. The RT-PCR experiments that have been performed later agree with this result, as it seems that StfZ RNA extends further than such a termination site would allow (Fig. 16, p. 61).

The insert of the plasmid pT20 is the same as that of the plasmid pP8, which was used in earlier experiments to search for the stfZ promoter. It exhibits strong termination activity. The insert of the plasmid pT21 is half the length of the insert of pT20 and shows much weaker termination activity. In the experiments described in section 3.2, ten different DNA fragments (350 bp or longer) from the mra gene cluster of E. coli chromosome were tested for termination activity in the antisense direction. Every one of these fragments had strong termination activity, possibly rho-dependent. Signals for rho-dependent termination are not very stringent and could be present in all of these fragments. Furthermore, their transcripts are not translated, which seems to be the most important condition for rho-dependent termination. Thus it is interesting that the insert of the plasmid pT21 has only weak termination activity, even though it is certainly long enough (623 bp) for rho-dependent termination, and the sequence analysis shows that its transcript is not likely to be translated. It seems as if the apparently ubiquitous signals for rho-dependent termination are absent or at least imperfect in the pT21 insert.

More constructs need to be made to find the exact location of the stfZ terminator. The GC-rich 8 bp inverted repeat mentioned earlier is the only feature of the sequence that has a resemblance to a rho-independent terminator. However, in this case there seems to be no necessity for rho-independent termination, since StfZ RNA is not translated and rho-dependent termination would work. If stfZ does have a rho-dependent terminator though, it could be very difficult to locate it precisely.
Table 3. Summary of the Plasmids and Their Insert Sizes

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Length (bp)</th>
<th>Galactokinase</th>
</tr>
</thead>
<tbody>
<tr>
<td>pT16</td>
<td>456</td>
<td>-</td>
</tr>
<tr>
<td>pT17</td>
<td>79</td>
<td>+</td>
</tr>
<tr>
<td>pT18</td>
<td>90</td>
<td>+</td>
</tr>
<tr>
<td>pT19</td>
<td>110</td>
<td>+</td>
</tr>
<tr>
<td>pT20</td>
<td>1259</td>
<td>-</td>
</tr>
<tr>
<td>pT21</td>
<td>623</td>
<td>+/-</td>
</tr>
</tbody>
</table>

Fig. 18. A search for stfZ terminators. The fragments shown here have been cloned in the indicated direction between the promoter P_gal and the galK gene in the termination assay vector pJW30 (Fig. 7, p. 36; Table 3, p. 37). The inserts of pT16 and pT20 both have strong termination activity. The insert of pT21 has weak termination activity. The presence of galactokinase activity was determined by the colour of E. coli C600K (galK) cells carrying the constructed plasmids when grown on MacConkey plates containing galactose.
5 The function of StfZ

5.1 Introduction

The experiments described in the previous chapter show that there is a gene in the antisense DNA strand of *E. coli* chromosome at the *ftsA* – *ftsZ* gene junction. It now has to be determined what the function of this gene is.

It seemed most likely that the product of *sfZ* is an antisense RNA, but the possibility that the transcript of *sfZ* might be translated into a protein could not be overlooked. To resolve this question, the sequence of the antisense strand at the *ftsA* – *ftsZ* gene junction (from −371 to +905 relative to the first nucleotide coding for FtsZ protein in the sense strand of DNA) was analysed by the program GeneMark™ (http://opal.biology.gatech.edu/GeneMark/), which is designed for predicting protein-coding regions in microbial DNA sequences. This program estimates probabilities of ribosomal binding site, start codon, and codon preferences for each open reading frame it finds in the sequence [35]. The results provided by GeneMark™ indicate that it is very unlikely there is a protein coded by the antisense strand of the DNA sequence examined. The probability is less than 0.1 for the sequence covering the putative *sfZ* location (as determined by RT-PCR experiments, section 4.2.2). The other two open reading frames in the region, extending outside the putative *sfZ* location, have probabilities of encoding a protein of 0.14 and 0.16 respectively. Therefore it is almost certain that the final product of *sfZ* is an RNA and not a protein.

We assume then that the StfZ RNA functions as an antisense RNA. The RT-PCR experiments described in section 4.2.2 indicate that the StfZ RNA is about 420 nt long. Most bacterial antisense RNAs discovered so far are quite short, 43 – 350 nucleotides [294, 302]. There seems to be no reason though why an antisense RNA could not be longer. It might also be that StfZ is processed after transcription and that the active antisense RNA is a shorter version of StfZ.

The ability of any antisense RNA to act very much depends on its secondary structure. The first step of interaction between an antisense RNA and a target RNA is a kissing reaction, when the two RNAs form base pairs between complementary sequences that are unpaired within each RNA, such as sequences in loops or
unstructured ends of RNA. After a kissing reaction, pairing usually propagates to form a stable duplex that can inhibit either transcription or translation of the target RNA. Stem-loop structures are important for antisense RNAs. The loops establish the interaction specificity, while the stems are involved in determining the stability of the RNA, and also influence the propagation of pairing between the two RNAs. It has also been shown that irregularities in a stem structure can be important in RNA-protein interaction, as in the case of the antisense control of plasmid ColE1 replication, where the protein Rop is needed to facilitate the interaction between the antisense and target RNAs [202].

The sequence of StfZ (423 nt long, confirmed by RT-PCR experiments, section 4.2.2) was used in the Michael Zuker program mfold version 3.1 [186] (http://bioinfo.math.rpi.edu/~mfold/rna/form1.cgi) to determine a possible secondary structure of StfZ (Fig. 19). The resulting structure is not very reliable since the mfold program folds an RNA sequence according to the energy values of the final RNA structure, while in vivo RNAs fold at the same time as they are produced, so the 5' end of an RNA starts folding first and can make base pairs with an adjacent region before another, possibly more complementary, region towards the 3' end of the RNA is transcribed and available for pairing. Also, the predicted structure might be very different if an RNA sequence of a different length would be used. As the exact length of StfZ is not known, this makes the structure prediction even less reliable.

The mfold program predicts several alternative structures with similar free energy. All such possible structures of StfZ have a stem-loop with the stem formed by the 8 bp inverted repeat of the fisA-ftsZ intergenic region (marked with a yellow area in Fig. 19). mfold predictions indicate that the ftsZ mRNA forms a matching stem-loop where the stem is formed by the 8 bp inverted repeat of the fisA-ftsZ intergenic region. The loop of this structure is complementary to the corresponding loop of the StfZ RNA and one could speculate that these two could be involved in the kissing reaction.
Fig. 19. A possible secondary structure of StfZ (predicted by *mfold* version 3.1 [186]). A stem-loop structure formed by the 8 bp inverted repeat in the *ftsA-ftsZ* intergenic region is indicated by a yellow area. A region complementary to Shine-Dalgarno sequence of the *ftsZ* mRNA is indicated by a red area.
necessary to initiate the interaction between an antisense RNA and a target RNA. If such interaction would indeed occur and spread, a nearby Shine-Dalgarno region of \( ftsZ \) mRNA could be bound by the complementary region of StfZ. This would make it impossible for ribosomes to bind the ribosomal binding site of \( ftsZ \) mRNA and so it would stop the translation of the \( ftsZ \) mRNA. This is, of course, purely a theoretical possibility and it will need to be tested by experiments to see if this is what happens \textit{in vivo}.

### 5.2 StfZ mutagenesis

Mutating or deleting the \( stfZ \) gene on the \( E. coli \) chromosome would be a good way to study the function of the StfZ RNA, for it might show what effect StfZ has on \( E. coli \) cells \textit{in vivo}. Specific mutations could help find the bases of StfZ responsible for interaction with \( ftsZ \) mRNA (if such interaction exists), and maybe indicate the mechanism of the interaction. The problem with such an approach in this case is that \( sfZ \) is on the opposite strand of the same stretch of DNA that contains the \( fisZ \) gene. Therefore, any change in \( sf/Z \) sequence might have an effect on \( ftsZ \) too. It is very likely that \( fisZ \) mRNA is the target of the StfZ RNA, so it would be difficult to distinguish the cause of any effect that a mutation affecting both \( fisZ \) and \( stfZ \) would have on \( E. coli \) cells. One could mutate some bases inside the \( ftsZ \) coding region while making sure that it still codes for the same amino acids, but that would not change complementarity between \( flsZ \) mRNA and StfZ, and so would probably not interfere with the function of StfZ. The best strategy would be to introduce mutations that would change the secondary structure of the RNAs. That could be done in the \( ftsA-fisZ \) intergenic region, and the 8 bp inverted repeat (Fig. 20) seems like a good place to start, as it is important for the secondary structures of both the \( fisZ \) mRNA and StfZ. It is also located outside the Shine-Dalgarno sequence of the \( fisZ \) mRNA, therefore initiation of translation would probably not be affected. On the other hand, the 8 bp inverted repeat is at the side of an RNase E cleavage site that has been shown to be important for degradation of the \( fisZ \) mRNA [47], and it is possible that weakening the stem formed by the 8 bp inverted repeat could interfere with RNase E recognition of the sequence and so influence the expression of \( fisZ \).
Despite this possible problem, I have attempted to introduce mutations into the 8 bp inverted repeat of the *ftsA*–*ftsZ* intergenic region. This would disrupt the stem formation and so would change the secondary structure of the StfZ RNA produced in the cells. A gene replacement vector pKO3 [163] (Fig. 7, p.36) was used to transfer the mutations into the *E. coli* MG1655 chromosome. A fragment of DNA containing the 3' end of *ftsA*, the *ftsA*–*ftsZ* intergenic region that has five changed bases (GGCCG into TCTTC, Fig. 20), and the whole *ftsZ* gene has been cloned into pKO3 to produce the plasmid pM2 (Fig. 21).

**Fig. 20.** The location of an 8 bp inverted repeat (indicated by two long arrows) in the *ftsA*–*ftsZ* intergenic region. A grey shaded box indicates the bases selected for mutation in *E. coli* MG1655-M2 (see text below), with bases that replace them written underneath in red. A black triangle shows an RNase E cleavage site. SD, *ftsZ* Shine-Dalgamo sequence.

**Fig. 21.** Plasmid pM2. The fragment containing the 3’ end of *ftsA* has been produced by PCR, using primers prim21-1 and prim21-2 (Table 4, p. 38), then cut by DNA endonucleases *NotI* and *Bsu36I*. The fragment containing *ftsZ* has been cut out of the plasmid pZAQ [301] using DNA endonucleases *Bsu36I* and *NgoMl*. The vector pKO3 (Fig. 7, p. 36) has been cut by *NotI* and *NgoMl*. 
Fig. 22 shows the strategy of mutagenesis used to make *E. coli* MG1655-M2 strain. The plasmid pM2 contains a temperature-sensitive origin of replication, so at 42°C it can be replicated in cells only if integrated into the chromosome. This can happen through homologous recombination at the *ftsA* or *ftsZ* genes. At lower temperatures, pM2 can recombine out of the chromosome and start replicating from its own origin. This event can restore the chromosome to its unmodified state or, preferably, retain the mutated *ftsA* – *ftsZ* intergenic region inside the chromosome and expel the original one into the plasmid. The *sacB* gene in the plasmid pM2 facilitates selection for the cells that have lost the plasmid – cells harbouring pM2 can not grow on media containing sucrose. In the experiments, the plasmid pM2 was electroporated into *E. coli* MG1655 cells. The cells were grown at 42°C to integrate pM2 into the chromosome. After that, the cells were grown at 30°C on LB plates containing sucrose to allow excision of pM2 from the chromosome and to select the cells that have lost the plasmid. The *ftsA* – *ftsZ* intergenic region of the chromosome from selected cells was sequenced to see if any of the cells retain the mutation. Sixteen different colonies were checked, but none of them had this mutation.

The “arms” to the left and to the right of the mutation in the insert of the plasmid pM2 are of different lengths, being 490 bp and 1376 bp respectively, and therefore the homologous recombination events have a greater probability of happening in the right “arm”. This means that quite often the crossover for both integrating pM2 into the chromosome, and removing it, would happen in the right “arm”. As a result the mutation will remain in the plasmid, and not be transferred into the chromosome. Still, the fact that none of the sixteen different colonies had the mutation inside the chromosome might indicate that the mutations were harmful – indeed the mutations could have even been lethal to the cells, though the small sample size does not allow one to draw definite conclusions.
Fig. 22. StfZ mutagenesis. Plasmid pM2 was electroporated into E. coli MG1655 cells. The cells were allowed to recover for 1 hour at 30°C, then plated on LB plates containing chloramphenicol and incubated at 42°C. Several of resulting colonies were diluted in LB medium and plated on LB plates containing 5% sucrose, then incubated at 30°C. The loss of the plasmid pM2 was confirmed by growing the colonies on LB plates with chloramphenicol. The ftsA – ftsZ intergenic region of the chromosomes from cells of different colonies was sequenced to test for the presence of the mutation.
5.3 Plasmids pS4 and pAG1

Another way to determine the effect of StfZ on cells would be to overexpress StfZ. The main problem with such an approach is that the exact ends of the StfZ RNA are still not known. For this reason, a plasmid was created that overexpresses a hybrid RNA that includes a part of the stfZ RNA. In this plasmid, pS4, a 206 bp fragment of DNA covering the putative site of the stfZ gene is cloned downstream of P_{lac} and 79 bases of the lacZ mRNA in the BamHI site of a cloning vector pUC18 (Fig. 23, Fig. 24, and Fig. 25). The 206 bp fragment was selected using the primer extension experiment results to predict the 5' end of stfZ (section 4.2.1), and using a terminator predicted by Dewar and Donachie [80] as the terminator of stfZ. Later, RT-PCR experiments have shown that stfZ extends further in both 5' and 3' directions than the fragment chosen for cloning (section 4.2.2). In the plasmid pS4, the RNA that is transcribed from P_{lac} contains some sequences that are not present in StfZ, so it is possible that it folds differently from StfZ, and its properties (such as stability) could be different from the StfZ RNA. This has to be remembered when evaluating the results of experiments using pS4, but such experiments could still help us in uncovering the function of StfZ.

![Diagram of plasmids pS4 and pAG1](image)

**Fig. 23.** The insert of plasmids pS4 and pAG1 is shown in red. The dotted grey line shows the putative location of the stfZ gene, as determined by RT-PCR experiments (section 4.2.2).
Fig. 24. Plasmids pS4 and pAG1. 

pS4 was created by inserting a 206 bp long DNA fragment covering the putative site of the \textit{sttZ} gene (Fig. 23) into the \textit{BamHI} site of a cloning vector pUC18. The 206 bp fragment was made by PCR (primers prim5-1 and prim6 were used, Table 4, p. 38; plasmid pZAQ was used as a template) – this allowed the introduction of \textit{BamHI} sites at the ends of the fragment. The insert site of the plasmid pS4 has been sequenced to verify the sequence and to determine the direction of the insert. The sequence is shown in Fig. 25. 

pAG1 was created by inserting a fragment containing a \textit{cat} gene into the \textit{Sspl} site of pS4. The fragment was made by PCR, using primers NewCat-dn and NewCat-up (Table 4, p. 38) and vector pBR325 [230] as a template. The PCR product was cut by \textit{Ecll}3611 and \textit{SmaI} to get blunt ends for ligation.

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Fig. 25. The sequence of the insert of plasmids pS4 and pAG1. The sequence sandwiched between two lines comes from the cloning vector pUC18. -35 and -10 indicate the \textit{lac} promoter. +1 indicates the start of transcription for the \textit{lacZ} mRNA. \( \rightarrow \) indicates the start of translation: the vector pUC18 includes a ribosomal binding site and a few starting codons of \textit{lacZ}, therefore a peptide of 41 amino acids can be produced from the hybrid RNA transcribed from \textit{P}_{\text{lac}} in pS4 or pAG1 (the stop codon for such a peptide is indicated here in bold letters). Two long arrows indicate an 8 bp inverted repeat. A black box indicates the bases complementary to the Shine-Dalgarno sequence of \textit{ftsZ}. 

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To see if the expression of the pS4 encoded antisense RNA is dependent on $P_{\text{lac}}$, a plasmid pVH1 [124] was introduced into the *E. coli* cells harbouring pS4. The plasmid pVH1 overexpresses the LacI protein which suppresses transcription from $P_{\text{lac}}$, but suppression can be alleviated by IPTG, thus allowing transcription from $P_{\text{lac}}$ to be controlled. pS4 in cells harbouring pVH1 had an effect (filamentation of cells, see section 5.4 below) only when IPTG was present, so it can be concluded that the pS4 encoded antisense RNA is expressed from $P_{\text{lac}}$.

The plasmid pS4 has a gene conferring resistance to ampicillin in cells that carry it, so ampicillin was used for selection and maintenance of pS4 in *E. coli* cells. Unfortunately, ampicillin is far from ideal as a marker since it is gradually hydrolysed by cells producing $\beta$-lactamase, and then at low concentrations it causes the cells without the resistance gene to filament. To get around this problem, a DNA fragment containing a chloramphenicol resistance gene was inserted into pS4 to produce the plasmid pAG1 (Fig. 24). The fragment was amplified by PCR using pBR325 [230] as a template and oligonucleotides NewCat-up and NewCat-dn (Table 4, p. 38) as primers. It was then cut by endonucleases EcoI136I and Smal and cloned into pS4 at SspI site. The plasmid pAG1 was used instead of pS4 in all of the subsequent experiments.

5.4 The effect of pAG1 on *E. coli* cells

As was described earlier, pAG1 (Fig. 24) is a plasmid constructed to overexpress the StfZ RNA, so that the effect of StfZ on cells could be assessed. Several *E. coli* strains were transformed with pAG1 and it was determined that, at high incubation temperatures, pAG1 inhibits cell division, lowers the concentration of FtsZ protein in cells, prevents FtsZ ring formation, and represses translation from the *ftsZ* ribosomal binding site.

5.4.1 Filamentation of cells

Plasmid pAG1 has been introduced into seven *E. coli* strains: MG1655, C600, MM38, SP5211, CSH26, HAT10, and DH5$\alpha$. In all these cases, the plasmid completely inhibits *E. coli* cell division when cells are grown at 42°C. At 30°C, however, the cells divide and are of normal size and shape (Fig. 26). At 37°C, the cells are longer than normal, depending on strain their length varies from several normal cell lengths to quite long filaments.
Cell filamentation is completely dependent on transcription from the $P_{lac}$ promoter in the plasmid pS4, as shown by experiments with cells carrying both pS4 and pVH1 plasmids (section 5.3). This should hold true also for the plasmid pAG1, since it is identical to pS4 except for an additional marker gene $cat$ (Fig. 24, p. 76).

Cells harbouring pAG1 are able to form colonies on LB plates at 42°C, even though the cells are filamenting. If the effect of pAG1 on cells were lethal and the cells would not be able to form colonies, then pAG1 could be used to search for mutations that interfere with StfZ action. We tried using cyclic AMP to enhance the effect of the pAG1 plasmid on cells, because cAMP upregulates transcription from $P_{lac}$. [262]. As expected, when cAMP is added to the growth medium, the cells harbouring pAG1 start to filament earlier than the cells grown without cAMP, and quite soon most of the filaments are dead. However, the cells that are grown on LB plates with cAMP are still able to form colonies, presumably from those few cells that are still alive when cAMP gets depleted from the growth medium. Instead of adding cAMP to the medium though, one could force cells to produce more cAMP by growing them on a minimal medium containing no glucose. When $E. coli$ cells harbouring pAG1 were grown on VBA plates at 42°C, they did not form colonies.

Cells carrying the pAG1 plasmid do not filament at low cell densities (up to about $OD_{600}$ of 0.3) when grown in liquid LB medium at 42°C. This could be due to the
effect of cAMP on the P_lac promoter in the plasmid pAG1. cAMP is a global regulatory factor involved in stationary phase induction of many E. coli genes. It also upregulates transcription from P_lac, so in stationary phase, the cells harbouring pAG1 should have increased production of the pAG1-encoded antisense RNA.

A frequent cause for cell division inhibition in E. coli is the SOS response. It is induced by damage to DNA leading to inhibition of its replication. An E. coli gene sfiA is induced as a part of the SOS response. SfiA inhibits FtsZ polymerisation and stops cell division [286]. To determine if the filamentation of the cells harbouring pAG1 is dependent on the SOS response, a strain with deactivated sfiA was used. E. coli MM38 sfiA::Tn5 cells carrying the plasmid pAG1, though deficient in SfiA function, filament when grown at 42°C, in the same way as E. coli MM38 cells carrying pAG1. Thus it can be concluded that filamentation of the cells harbouring pAG1 does not depend on the SOS response.

The E. coli gene rodA is important for cell shape determination [13]. The E. coli rodA mutant cells are round. It has been observed earlier that when division of rodA mutant cells is inhibited, they become lemon-shaped if the cell division is blocked at an early stage, for example by an FtsZ mutation. If the cell division is blocked at a later stage, for example by an FtsA mutation, a forming septum can be seen in the cells trying to divide [24]. In our experiments, E. coli C600 rodA ts cells carrying the plasmid pAG1 are lemon-shaped when grown at 42°C (not shown here), suggesting that division is stopped at an early stage. This is in agreement with the hypothesis that the StfZ RNA inhibits FtsZ production.

The plasmid pAG1 has the greatest effect on cells that are grown at high temperatures. The reason for this is not clear. It could be due to the changes in stability of secondary RNA structure at different temperatures, because secondary structure is very important for the action of antisense RNAs. The program mfold version 3.1 [186] was used to determine a possible secondary structure of the pAG1-encoded antisense RNA (Fig. 27). In most mfold predictions, the antisense RNA region complementary to the fisZ Shine-Dalgarno sequence is paired to another region of the antisense RNA. As mentioned before, mfold predictions are not very reliable (see section 5.1), and the structure shown in Fig. 27 has long-range interactions not very likely to form while RNA is transcribed. Prediction again is complicated by the fact that the 3' end of the pAG1-encoded RNA is not known.
Nevertheless, it still might be that some region of the pAG1-encoded antisense RNA needs to be unpaired to be able to bind the *ftsZ* mRNA. High temperature makes base pairing less rigid, and might be necessary for interaction between the pAG1-encoded antisense RNA and the *ftsZ* mRNA.

Fig. 27. A possible secondary structure of the pAG1-encoded antisense RNA (predicted by *mfold version 3.1* [186]). A region complementary to Shine-Dalgarno sequence of the *ftsZ* mRNA is indicated by a red area.
5.4.2 Inhibition of FtsZ production

To see if filamentation of the cells harbouring pAG1 can be due to inhibition of ftsZ expression, the cellular concentration of the FtsZ protein was assessed using Western blotting experiments. The *E. coli* MG1655 and MG1655 + pAG1 cells were grown in liquid LB medium at 30°C or 42°C until OD<sub>600</sub> reached 2. The MG1655 + pAG1 cells grown at 42°C were filamentous. All the other cells were of normal size. In order to isolate the proteins, crude cell extracts were made. The concentration of total proteins in crude cell extracts was measured by dotMETRIC™ 1μl Protein Assay (Chemicon). The crude cell extracts were run on several 13 % SDS-PAGE gels together with prestained Biolabs protein markers. Western blots of the gels using an anti-FtsZ antibody show that the concentration of the FtsZ protein in *E. coli* MG1655 + pAG1 cells grown at 42°C is about four times lower than in MG1655 cells grown at 42°C (Fig. 28, left). They also show that the plasmid pAG1 does not affect the levels of FtsZ when the cells are grown at 30°C (Fig. 28, right).

![Fig. 28. Western blots. Crude protein extracts from *E. coli* MG1655 and MG1655 + pAG1 cells were run in two 13 % SDS-PAGE gels, then transferred to nitrocellulose sheets, and hybridised to an anti-FtsZ antibody. Each lane in a single gel has approximately the same amount of total protein (the concentration of proteins was measured by dotMETRIC™ 1μl Protein Assay (Chemicon)). To prepare the protein extracts, the cells have been grown in LB medium until OD<sub>600</sub> reached 2. Left: lane 1, protein extract from MG1655 cells grown at 42°C; lanes 2-4, dilutions (×2, ×4, and ×8) of the protein extract in lane 1; lane 5, protein extract from MG1655 + pAG1 cells grown at 42°C. Right: lane 1, protein extract from MG1655 cells grown at 30°C; lane 2, protein extract from MG1655 + pAG1 cells grown at 30°C; lane 3, protein extract from MG1655 + pAG1 cells grown at 42°C. This demonstrates that the pAG1 plasmid reduces the concentration of the FtsZ protein in *E. coli* cells grown at 42°C.](image-url)
5.4.3 Inhibition of FtsZ ring formation

When *E. coli* cells harbouring the plasmid pAG1 are incubated at 42°C, they stop dividing, form long filaments, and eventually die. To see if the filaments have FtsZ rings (which are necessary, but not sufficient for cell division), *E. coli* C600 + pAG1 cells were immuno-stained with an anti-FtsZ antibody. In addition, two control strains were used: (1) *E. coli* C600 cells without the plasmid pAG1 that divide normally and should have FtsZ rings in all the cells that are going to divide; (2) *E. coli* C600 *ftsA*<sub>12</sub> cells filament at 42°C, but their division stops because of a defect in the essential cell division gene *ftsA*, so FtsZ ring formation is not affected.

The immuno-fluorescent staining experiments show that there are almost no FtsZ rings in *E. coli* C600 cells carrying the pAG1 plasmid when they are grown at 42°C (Fig. 29). In comparison, both C600 and C600 *ftsA*<sub>12</sub> cells have normal FtsZ rings (Fig. 29).

![Fig. 29. Immuno-fluorescent staining of FtsZ rings. *E. coli* C600, C600 *ftsA*<sub>12</sub>, and C600 + pAG1 cells were grown in liquid LB medium at 42°C until OD<sub>600</sub> reached approximately 0.5. The cells were fixed, permeabilised, treated with an anti-FtsZ antibody, and then with a fluorescent secondary antibody.](image-url)
5.4.4 Repression of translation from the ftsZ ribosomal binding site

One of the mechanisms of antisense RNA action involves the RNA binding to the ribosomal binding site (RBS) of the target RNA thus preventing the binding of a ribosome and the start of translation. To check if the plasmid pAG1 affects translation from the RBS of the ftsZ mRNA, E. coli strains TP8503 [λR2], TP8503 [λR5], and TP8503 [λR7] were constructed. They allow one to quantitatively measure the expression from the RBS of the ftsZ mRNA. In these strains, cell β-galactosidase activity is used as an indicator of such expression. For this reason, TP8503 was used as a parental strain – its native chromosomal lac locus is deleted. The strains TP8503 [λR2], TP8503 [λR5], and TP8503 [λR7] each have a single copy of one of the constructs described below.

The constructs inside E. coli TP8503 [λR2], TP8503 [λR5], and TP8503 [λR7] consist of (see also Fig. 30 and Fig. 31):

- The constitutive promoter P_rplK [227, 231], for initiation of transcription.
- The RBS from ftsZ or the λ gene E to initiate translation. The strain TP8503 [λR2] has the RBS of ftsZ and some sequence upstream of that. E. coli TP8503 [λR5] has the RBS of the λ gene E [249]. E. coli TP8503 [λR7] has the RBS of ftsZ, but not the upstream sequence present in TP8503 [λR2].
- An open reading frame (ORF) that codes for a fusion protein with β-galactosidase activity. ftsZ::lacZ is the ORF in the strains TP8503 [λR2] and TP8503 [λR7]. E::lacZ is the ORF in the strain TP8503 [λR5]. The basis for these open reading frames is the E. coli gene lacZ, minus its first eight codons. In ftsZ::lacZ, at the N-terminus ‘lacZ is fused to the first four ftsZ codons followed by two other codons that make a restriction site. In E::lacZ, at the N-terminus ‘lacZ is fused to the first 17 codons of the λ gene E followed by three other codons at the introduced restriction site.

The constructs are integrated into the E. coli TP8503 chromosome to ensure there is only one copy per cell. The strains TP8503 [λR2], TP8503 [λR5], and TP8503 [λR7] were constructed by the following steps: (1) the DNA fragment containing the P_rplK promoter was amplified by PCR using primers prim16-2 and prim16-3 (Table 4, p. 38) and the plasmid pHR10 as a template (pHR10 is a pBR322 derivative plasmid that carries a 260 bp Sau3A-EcoRI fragment containing E. coli P_rplK). The PCR
product was cut by endonucleases *EcoRI* and *BamHI* and ligated into the vector pRS552 [261] (Fig. 7, p. 36) at the *EcoRI*-*BamHI* site to make a plasmid pR1. (2a) A 146 bp DNA fragment containing the RBS of *fisZ* was amplified by PCR using primers prim17-1 and prim17-2 (Table 4, p. 38) and the plasmid pZAQ [301] as a template. The PCR product was cut by *BamHI* and ligated into the plasmid pR1 at the *BamHI* site to produce a plasmid pR2. (2b) The DNA fragment containing the RBS of the λ gene *E* was amplified by PCR using primers prim25-1 and prim25-2 (Table 4, p. 38) and λ DNA as a template. The PCR product was cut by *BamHI* and ligated into the plasmid pR1 at the *BamHI* site to produce a plasmid pR5. (2c) A 38 bp DNA fragment containing the RBS of *ftsZ* was assembled by hybridising two oligonucleotides:

5′ GAT CCA GGC ACA AAT CGG AGA GAA ACT ATG TTT
GAA CCA ATG and 5′ GAT CCA TTG GTT CAA ACA TAG TTT CTC TCC
GAT TTT TGC CTG. The hybridised DNA fragment was ligated into the plasmid pR1 at the *BamHI* site to produce a plasmid pR7. (2d) The inserts of the plasmids pR1, pR2, pR5, and pR7 were sequenced to check if the orientation and the sequence of the inserts were correct. (3) The fusion constructs in the plasmids pR2, pR5, and pR7 were transferred by homologous recombination into a λ phage vector λRS45 [261] to make phages λR2, λR5, and λR7. (4) *E. coli* TP8503 cells were infected with the phages to produce the strains TP8503 [λR2], TP8503 [λR5], and TP8503 [λR7]. (5) The lysogens were screened to make sure there was only one copy of the construct integrated into a chromosome.

![Fig. 30](image_url)

**Fig. 30.** The constructs inserted into *E. coli* TP8503 [λR2], TP8503 [λR5], and TP8503 [λR7] chromosomes. *P pik*, a constitutive promoter (normally in front of a gene coding for *E. coli* ribosomal protein L11); RBS, a ribosomal binding site; *E*, λ gene *E*; *ftsZ::lacZ* and *E::lacZ*, open reading frames coding for fusion proteins with β-galactosidase activity (see text). The sequences of the constructs are shown in Fig. 31.
In the experiments, the TP8503 [λR5] strain was used as a control. Its β-galactosidase activity has proved to be much lower than that of strains TP8503 [λR2] and TP8503 [λR7]. It is not clear whether this is because the RBS of λ gene E is much less effective at initiation of translation than the RBS of ftsZ, because of the difference in β-galactosidase activity of the fusion proteins FtsZ::LacZ and E::LacZ, or because the stability of RNA is affected. Either way, TP8503 [λR5] is unfortunately not a perfect control.

In the TP8503 [λR2] strain, the RNA produced from the PrplK promoter has an RNase E cleavage site (Fig. 31). It has been reported that this RNase E cleavage site is important for the stability of the ftsZ mRNA [47], so it might influence the rate of degradation of the produced hybrid RNA as well. To eliminate its effect on
expression of the fusion protein FtsZ::LacZ, the strain TP8503 [λR7] was constructed. It has a short sequence corresponding to the ftsZ RBS and does not have the RNase E cleavage site.

The *E. coli* strains TP8503 [λR2], TP8503 [λR5], and TP8503 [λR7] were transformed by the plasmid pAG1 and grown in LB medium at 30°C, 37°C and 42°C. The strains without the plasmid were also grown at the same conditions to be used as controls. β-galactosidase assays were performed to find out the differences in expression of the fusion proteins FtsZ::LacZ or E::LacZ from the inserted RBSs. The results are shown in Figures 32-34. At 30°C, the plasmid pAG1 did not have a significant effect on the expression of ftsZ::lacZ or E::lacZ. At 42°C, pAG1 strongly inhibits the expression of ftsZ::lacZ in both the TP8503 [λR2] and TP8503 [λR7] strains but not the expression of E::lacZ in the control TP8503 [λR5] strain. At 37°C, the effect of pAG1 on the expression of ftsZ::lacZ is intermediate.

The inhibition of ftsZ::lacZ expression by pAG1 in the TP8503 [λR7] strain is stronger than in the TP8503 [λR2] strain. This demonstrates that the RNase E site located upstream of the RBS of ftsZ in the TP8503 [λR2] strain is not necessary for the pAG1-mediated inhibition. Moreover, the 8 bp inverted repeat that is located immediately downstream from the RNase E site and that probably forms a stem-loop in the *ftsZ* mRNA is not required for the pAG1-mediated inhibition of ftsZ::lacZ expression and might even interfere with such inhibition.

The plasmid pAG1 does not reduce the expression of E::lacZ in the control strain TP8503 [λR5]. This indicates that pAG1 does not inhibit transcription from the *rplK* promoter. Therefore, the results from the β-galactosidase assays show that at high temperatures pAG1 inhibits translation from the RBS of *ftsZ*.
Fig 3. β-galactosidase assays showing expression of the FtsZ::LacZ and E::LacZ fusion proteins in *E. coli* strains TP8503 [λR2], TP8503 [λR5], and TP8503 [λR7], and the effect that the plasmid pAG1 has on the expression. The cells were grown at 30°C in LB medium.
Fig 33. β–galactosidase assays showing expression of the FtsZ::LacZ and E::LacZ fusion proteins in E. coli strains TP8503 [λR2], TP8503 [λR7], and TP8503 [λR5], and the effect that the plasmid pAG1 has on the expression. The cells were grown at 37°C in LB medium.
Fig. 34. β-galactosidase assays showing expression of the FtsZ::LacZ and E::LacZ fusion proteins in *E. coli* strains TP8503 [λ.R2], TP8503 [λ.R7], and TP8503 [λ.R5], and the effect that the plasmid pAG1 has on the expression. The cells were grown at 42°C in LB medium.
5.4.5 Conclusions

The experiments with the plasmid pAG1 can not show conclusively what effect the natural StfZ RNA has on *E. coli* cells. As was mentioned earlier, this is because the pAG1-encoded antisense RNA is a hybrid RNA and does not contain the entire sequence of StfZ. However, the pAG1-encoded antisense RNA does include 206 nt of the StfZ RNA, and it acts on the ribosomal binding site of the *ftsZ* mRNA, the proposed target of StfZ. The function of the StfZ RNA could be to regulate expression of *ftsZ*, by binding to the RBS of the *ftsZ* mRNA and inhibiting its translation. The results of all the experiments using pAG1 confirm that this might be the case, even if they do not prove it.

5.5 The effect of natural StfZ on *E. coli* cells

The *E. coli* strains TP8503 [λR2], TP8503 [λR5], and TP8503 [λR7] were designed to determine the effect the plasmid pAG1 has on translation from the ribosomal binding site of *ftsZ* mRNA (section 5.4.4, p. 83). In the experiments, β-galactosidase activity of the strains not carrying the plasmid pAG1 was also measured, to be used as a control. The results revealed that, even without the plasmid, expression from the RBS of *ftsZ* is regulated.

β-galactosidase assays show that specific β-galactosidase activity in the strains TP8503 [λR2] and TP8503 [λR7] increases greatly as cells approach and enter the stationary phase, especially at high temperatures (Fig. 35 and Fig. 36). Specific β-galactosidase activity in the control strain TP8503 [λR5] does not vary significantly with the growth phase (Fig. 35 and Fig. 36), which indicates that the regulation of the fusion protein FtsZ::LacZ expression is not at a promoter level. Therefore, there has to be a regulator (such as the StfZ RNA, for example) in the cells that controls translation from the RBS of *ftsZ* in such a way that translation levels are the lowest at the exponential growth phase. This would make FtsZ protein concentration in exponentially growing cells lower than the concentration in cells in the stationary growth phase. Exponentially growing cells are larger than the cells in the stationary phase, so they divide less often per cell mass. Since they need the same amount of FtsZ protein per division, they require a lower concentration of FtsZ than
Fig. 35. β-galactosidase assays showing expression of the FtsZ::LacZ and E::LacZ fusion proteins in *E. coli* strains TP8503 [λR2], TP8503 [λR5], and TP8503 [λR7]. The cells were grown in LB medium. Before the start of the measurements, the cultures were grown at 30°C overnight, then diluted 100x into LB medium, grown at 30°C, 37°C, or 42°C, until cell density reached OD₆₀₀ of about 0.3, then diluted and grown three more times to maintain the cultures at low density.
Fig. 36. β-galactosidase assays. Here, the data represented in Fig. 35 is normalised (β-galactosidase activities of the first cell culture samples (at OD_{600} ~ 0.1) were given the value of 1, and the activities of the rest of the samples were divided by the original values of the first samples).
the cells in stationary growth phase. The effect of such translational regulation of FtsZ levels is similar to the effect achieved by regulation of \( ftsZ \) transcription from gearbox promoters [4].

As was pointed out earlier, the construct inside the TP8503 [\( \lambda R2 \)] chromosome contains an RNase E cleavage site upstream of the RBS of \( ftsZ \) that is believed to influence the rate of \( ftsZ \) mRNA degradation [47]. The strain TP8503 [\( \lambda R7 \)] was constructed to exclude this RNase E cleavage site (Fig. 31, p. 85). Experiments show that the specific \( \beta \)-galactosidase activity of the TP8503 [\( \lambda R7 \)] cells is much higher than that of the TP8503 [\( \lambda R2 \)] cells (Fig. 35). This indicates that the RNase E site might indeed be important in determining stability of the \( ftsZ \) mRNA and so in modifying \( ftsZ \) expression. It is still possible that the stability of RNA in these two constructs differs because of reasons other than the RNase E cleavage.

Expression from the RBS of \( ftsZ \) depends on the growth phase of cells much more in the TP8503 [\( \lambda R2 \)] than in the TP8503 [\( \lambda R7 \)] strain (Fig. 36). In comparison with TP8503 [\( \lambda R2 \)], the TP8503 [\( \lambda R7 \)] strain lacks a GC-rich 8 bp inverted repeat as well as the RNase E cleavage site (Fig. 31, p. 85). There could be several explanations for the difference that the growth phase has on expression: (1) if the post-transcriptional regulator of \( ftsZ::lacZ \) expression is an antisense RNA, the inverted repeat could be important for binding of the antisense RNA to the \( ftsZ \) mRNA or to the hybrid \( ftsZ::lacZ \) mRNA. (2) The hybrid \( ftsZ::lacZ \) mRNA might become a better substrate for regulation by the antisense RNA after it has been cut by RNase E. (3) Perhaps RNase E can regulate the expression from \( ftsZ::lacZ \) mRNA, but this is unlikely since there is no evidence that RNase E activity in cells changes with growth phase. (4) The TP8503 [\( \lambda R7 \)] strain has higher specific \( \beta \)-galactosidase activity than the TP8503 [\( \lambda R2 \)] strain. It is possible that RNase cleavage makes the hybrid \( ftsZ::lacZ \) mRNA from the TP8503 [\( \lambda R2 \)] strain less stable than the hybrid mRNA from TP8503 [\( \lambda R7 \)]. This would make the concentration of the hybrid mRNA containing the \( ftsZ \) RBS higher in TP8503 [\( \lambda R7 \)] than in TP8503 [\( \lambda R2 \)], so there would be fewer antisense RNA molecules per \( ftsZ \) ribosomal binding site, and the effect the antisense
RNA has on overall expression of the FtsZ::LacZ fusion protein would be less prominent.

5.5.1 Conclusions
The β-galactosidase experiments using the strains TP8503 [λR2], TP8503 [λR5], and TP8503 [λR7] provide evidence that there is post-transcriptional regulation of protein expression from the fisZ ribosomal binding site in E. coli cells. The regulation is inversely dependent on growth phase of cells. Neither the GC-rich 8 bp inverted repeat nor the RNase E cleavage site are necessary for this post-transcriptional regulation, but one or both of them might facilitate it.

5.6 Possibility of other factors required for StfZ function
It is an interesting fact that pAG1, a plasmid that has been constructed to overexpress StfZ RNA, has the greatest effect on FtsZ production when E. coli cells are incubated at high temperatures (section 5.4). Another antisense RNA that targets fisZ, DicF, has also been shown to work better at high incubation temperatures [36]. Since 42°C is not a usual temperature for E. coli cells to find themselves in, it raises a question of whether there is some protein factor that facilitates the interaction between the fisZ mRNA and an antisense RNA, allowing such interaction at lower temperatures. Such a hypothesis is somewhat supported by the observation that the extreme temperature-dependency of the effect that pAG1 has on fisZ translation is not apparent in experiments with cells that do not harbour pAG1 but have only the natural StfZ expressed in them (section 5.5). In this case, the interaction between the natural StfZ and the fisZ mRNA would presumably have enough of the required factor available, but when the overexpressed antisense RNA is added, there might not be enough of the factor to help the interaction. Alternatively, the temperature dependency might be explained by the fact that the antisense RNA produced by pAG1 is not identical to StfZ, thus it could naturally require a higher temperature for action.

To find if a protein factor is needed for interaction between the fisZ mRNA and StfZ, transposon mutagenesis was used. E. coli cells carrying the plasmid pAG1 do not form colonies on VBA plates when incubated at 42°C. If introduction of a transposon somewhere in the chromosome could make cells form colonies on VBA plates at
42°C, that would mean the transposon is probably interfering with the action of StfZ. In such case the transposon might be inserted into a gene coding for a factor required for interaction between StfZ and \textit{fisZ} mRNA, and the gene would be easy to locate.

\textit{E. coli} W3110 + pAG1 cells were infected with phage \( \lambda \text{NK1316} \) [306]. The phage carries a mini-transposon Tn10, and it also has an amber mutation in an essential gene. \textit{E. coli} W3110 does not have amber suppressors, so \( \lambda \text{NK1316} \) cannot replicate there, and it cannot integrate into the chromosome either. The infected cells were plated on LB plates with ampicillin, chloramphenicol, and kanamycin and incubated at 30°C (pAG1 confers resistance to ampicillin and chloramphenicol, mini-Tn10 confers resistance to kanamycin). Approximately 25,000 resultant colonies were transferred to VBA plates with the same three antibiotics and incubated at 42°C. There were no colonies formed on these plates. It is unlikely that any chromosomal genes have been missed by the mini-transposon. The \textit{E. coli} chromosome is about 4,640,000 bp long, so, assuming the transposons have been integrated into the chromosome randomly, 25,000 different colonies would have the transposon inserted into sites roughly 200 bases apart.

There can be several explanations for the negative results: (1) no extra factors are required for StfZ function; (2) StfZ function requires a factor only at low temperatures, not at 42°C; (3) the antisense RNA overexpressed by pAG1 functions differently from StfZ and does not require a factor; (4) the factor is essential for cell survival, so if it is disrupted by a mini-Tn10, the cell dies.

Since pAG1 has the greatest effect on FtsZ expression at high temperatures, it seemed worthwhile to check if the interaction between the antisense RNA coded by pAG1 and the \textit{fisZ} mRNA is aided by any of the heat shock factors. To test this, ethanol was used in a growth medium to induce heat shock response in cells [290]. \textit{E. coli} C600 cells carrying the pAG1 plasmid were grown in VBB medium with ampicillin and chloramphenicol, at 30°C, until their density reached \( \text{OD}_{600} \) of 0.4. Then ethanol was added to the medium (to make a final ethanol concentration of 4%) and the cells continued to grow until \( \text{OD}_{600} \) of 1.6. The appearance of the cells was
normal, and they did not filament. This indicates that the function of the antisense RNA does not depend on heat shock factors (or at least that they are not sufficient).

Hfq (host factor I) is another protein that could be considered for the role of a factor required for StfZ – ftsZ mRNA interaction. Hfq can bind DNA and RNA molecules [256, 273]. It is needed for efficient translation of the stationary phase sigma factor RpoS [44, 204], and it regulates production of the outer membrane iron transport proteins FepA and FhuE in an rpoS-independent manner [293]. It has also been reported that the E. coli hfq::cat mutant strain produces increased amounts of FtsZ in the stationary phase [274].

To test if Hfq affects the interaction between the ftsZ mRNA and the antisense RNA produced by the plasmid pAG1, E. coli strain CSH26 and its derivative HATIO (hfq::cat) were transformed by pAG1 and grown in LB medium at different temperatures. There was no difference in filamentation between the CSH26 + pAG1 and HATIO + pAG1 strains. Since it is likely that at 42°C the antisense RNA from pAG1 can cause filamentation without help from any other factors, the result is not very surprising. It would be interesting to overexpress Hfq in order to see if it makes any difference for the effect the pAG1 plasmid has on E. coli cells. Also, the constructs P_{rptK} – RBS of ftsZ – ftsZ: :lacZ used in strains TP8503 [λR2] and TP8503 [λR7] could be transferred into strains CSH26 and HAT10. This might show whether the Hfq protein is needed for interaction between StfZ and ftsZ mRNA.
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