Localization of the ParC protein in *Escherichia coli* cells

Thesis submitted for the *cand. pharm* degree in microbiology

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ABSTRACT

The *Escherichia coli* ParC protein is one of two subunits of the topoisomerase IV, a type II topoisomerase. Topo IV is responsible for relaxing positive supercoils and decatenating linked daughter chromosomes after replication. This is done by introducing transient double-stranded breaks into the DNA, passing through a segment of uncut duplex DNA, before resealing the break. Mutations in *parC* lead to the par phenotype which is characterized by chromosomes that can replicate but are deficient in chromosomal partitioning. ParC is thus essential for cell survival. ParC has been found to associate with the replication machinery in the cell. ParE, Topo IV´s second subunit, was in the same study not found along with ParC. The difference in localization of ParC and ParE is proposed to underlie a temporal regulation of Topo IV´s activity in the cell. The activity of ParC may also be stimulated by SeqA, a protein preventing overinitiation of chromosome replication, and it is suggested that this stimulation is mediated by a specific interaction of Topo IV and SeqA.

In order to further look into ParC´s localization and function in the cell and to compare with SeqA´s assumed localization to the replication factory; both ParC and SeqA antibody were used in immunostaining followed by fluorescence microscopy. ParC antibody was, in opposition to SeqA antibody, not found to be localized as discrete foci and the co-localization of ParC protein to the replication factory is thus uncertain.

The ParC protein was purified in order to make an affinity column to purify the ParC antiserum used in the immunofluorescence microscopy. An expression plasmid was constructed by cloning the *parC* gene with a hexahistidine tag into a vector harbouring a T7 promoter. The expression plasmid was transformed into an *E. coli* strain that expresses T7 RNApolymerase. ParC protein was purified as a His-tagged protein by affinity chromatography based on the interaction between nickel and histidine, followed by gelfiltration.

ABBREVIATIONS

Ab	Antibody
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BSA	Bovine serum albumine
Da	Dalton
dH_2O	Distilled water
dNTP	Deoxynucleotide triphospate (ATP, GTP, CTP, TTP)
et al.	et alibi (with others)
EtBr	Ethidium bromide
F	Farad
g	Gravity
IgG	Immunoglobulin G
In vitro	Process acting in a reaction tube
In vivo	Process acting in a living cell
kb	Kilobases
(l)	Liquid
M	Molar
OD	Optical density
Ω	Ohm
PAGE	Polyacrylamide gelelectrophoresis
rpm	Rounds per minute
U	Enzyme unit
UV	Ultraviolet
V	Volt

PREFIXES

M	mega	10^{6}
k	kilo	10^{3}
m	milli	10^{-3}
μ	micro	10^{-6}
n	nano	10^{-9}
p	pico	10^{-12}

1 INTRODUCTION

The bacterium Escherichia coli belongs to the eubacteria group, one of two domains of prokaryotes in the living world. *E. coli* is a gram-negative, facultative anaerobe, non-spore-forming rod, ~1-2 μm wide and 3-30 μm long, with peritrichous flagella. It classifies under the Enterobacteriaceae family. The *E. coli* genome is 4.7 x 10^6 base pair, and is a circular double-stranded molecule (Blattner *et al.*, 1997). Most of the understanding of fundamental genetic mechanisms of life has come from studies of *E. coli*. Since some of these mechanisms are highly conserved throughout evolution, they are essentially the same in other prokaryotic and eukaryotic cells, and makes *E. coli* a commonly used model system.

1.1 The E. coli cell cycle

The cell cycle starts with DNA replication of the chromosome, then follows segregation of the daughter chromosomes and cell division by binary fission to form two identical daughter cells. Each daughter cell receives one copy of the cells genome.

The cell cycle is divided into several periods (Helmstetter, 2007). The I period, interinitiation time, is the time required to prepare for initiation of replication. During balanced growth the interdivision time (τ), which is the same as generation time, is equal to, and determined by the interinitiation time. The C period includes the chromosome replication, which initiates at *oriC*, elongates bidirectionally along the chromosome and terminates in *ter*. The period from termination of replication to the end of cell division is the D period.

The C and D periods are relatively constant, 40 minutes and 20 minutes respectively for cells with growth rates shorter than 60 minutes. For these cells the replication follows the

cell division (I = C+D). When duplication time exceeds 60 minutes (I > C+D), there is a period between cell division and initiation of replication termed the B period. Cells with doubling times above 60 minutes also have longer C and D periods (Skarstad *et al.*, 1983;Skarstad *et al.*, 1985). Depending on the availability of nutrients, *E. coli* are able to adjust the growth rate and thereby the generation time. In rich medium *E. coli* can have a duplication time of 20 minutes (I < C). One important issue that distinguishes fast-growing cells from slow-growing cells is when τ is less than C+D. Then there is more than one origin at the time of initiation of replication, and replication starts at all origins essentially simultaneously (Skarstad *et al.*, 1986). Since the C and D periods are relatively constant the initiation of replication must start before the previous round is completed. This results in a multiforked replication (Figure 1.1).

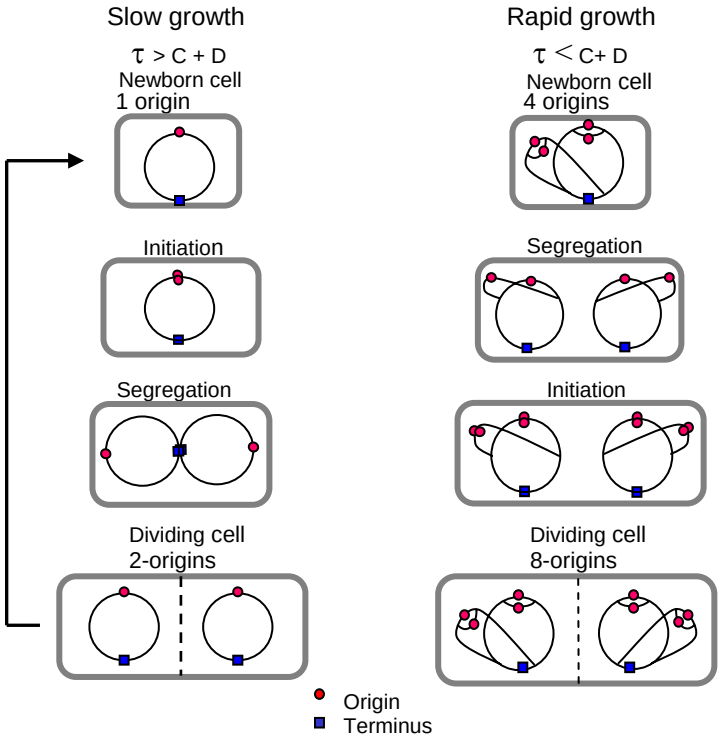


Figure 1.1 *The cell cycle of E. coli during slow and rapid growth. During slow growth the initiation of replication occurs at one single origin. The replication (C period) and cell division (D period) completes within one generation (τ >C+D) and result in dividing cell with 2 origins. During rapid growth the cell initiates at four origins. The C+D periods in this example take almost 3 generations, and the result is dividing cells with eight origins. The picture was obtained from Trond Bachs Doctoral Thesis, 2005*

1.2 Replication

The replication initiates in the *oriC*, which is located at 84.1 min on the genetic map. During replication, the strands in the DNA duplex are copied by base paring into two progeny identical duplexes (Kornberg and Baker, 1992).

1.2.1 Initiation of replication

In rapidly growing cells there are several copies of *oriC* due to multiforked replication. Initiation of the origins occurs simultaneously, only once per cell cycle and the cells have, at any time 2^n (n = 0, 1, 2, 3…) origins (Skarstad *et al.*, 1986).

The *oriC* region

In order to start replication the duplex needs to unwind and the strands separate. These topological alterations have a starting point in the origin. There are several repeating DNA segments which are critical for *oriC* function (figure 1.2). The highly conserved 9-mer motif which is the binding site for the initiator protein DnaA (R1-R5 (M)) is repeated five times and controls the unwinding of DNA (DnaA boxes). The I-sites, I1-I3, are also DnaA binding sites. The 13-mer motif, repeated three times in the AT rich region located in the left part of *oriC*, is the initial site of strand separation. Other repeating sequences in oriC are eleven GATC sites where Dam methyl transferase adds methyl groups. SeqA, also bind to the GATC sites and inactivates newly replicated origins (von Freiesleben *et al.*, 1994). In addition, there are binding sites for the DNA bending protein IHF, which assists in *oriC* unwinding, and the DNA bending protein Fis (Leonard and Grimwade, 2005).

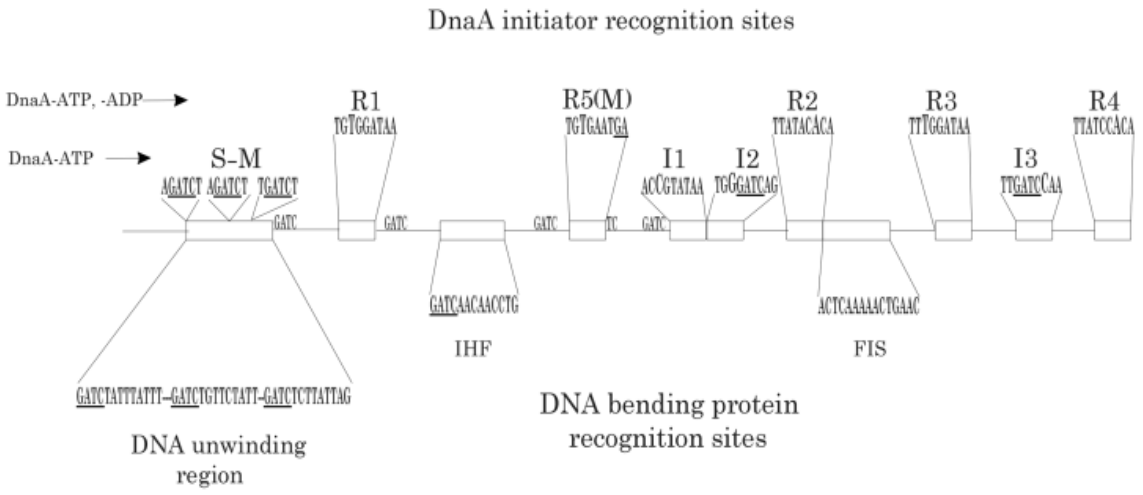


Figure 1.2 *E. coli replication origin, oriC. GATC sites are found at 11 locations (underlined), R1-R5 and I1-I3 are DnaA binding sites. There are also a binding site for the proteins Integration Host Factor (IHF) and Factor for Inversion Stimulation (FIS). The DNA unwinding region contains 3 repeats of an AT-rich 13-mer sequence. The picture was obtained from Leonard and Grimwade, 2004*

Molecular events of initiation at *oriC*

Upon initiation of replication the involved proteins assembles gradually at *oriC* and forms orisome complexes which together unwind DNA. When the initiator protein, DnaA is bound to ATP (not ADP), it interacts with DNA in the AT-rich region in the left part of *oriC* (figure 1.3). This interaction within the 13-mer region begins the unwinding of the DNA duplex and the initial complex is converted to an open complex.

DnaA recruits a complex of DNA helicase, DnaB, and helicase loader DnaC. Both DnaB and DnaC are hexameric rings and bind ATP, and release of DnaC activates DnaB by ATP hydrolysis. DnaG primase interacts with DnaB helicase, resulting in the priming complex. DnaG primase synthesizes two short RNA primers, the dimeric complex of DNA polymerase III holoenzyme binds to the primers, and sliding clamps (a ring-shaped β-subunit dimer of the DNA polymerase III) are assembled onto each primed template by a clamp loader (the polymerase III γ complex) (Kelman and O'Donnell, 1995) forming the replisome. DNA gyrase is required to relieve topological stress ahead of the replication forks, and together with SSB (Single-stranded DNA binding proteins) loaded, the replication can start (Messer and Weigel, 2007)

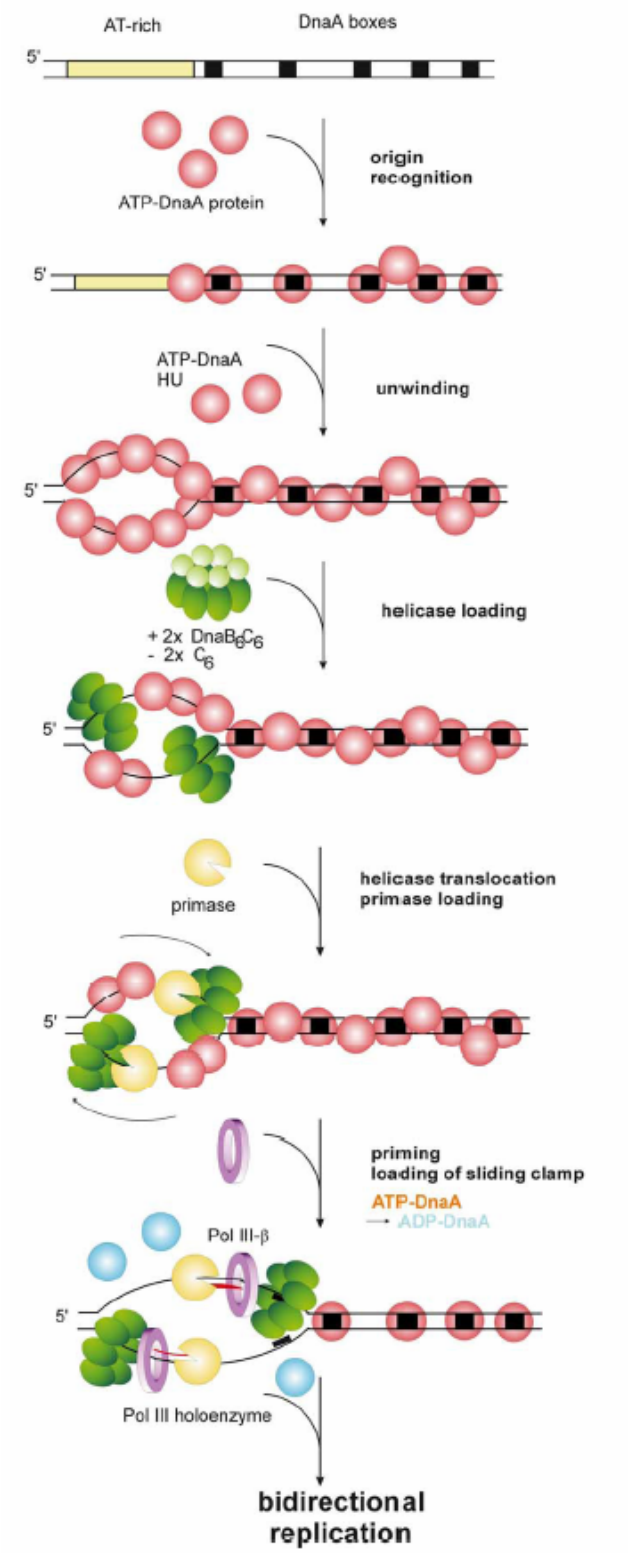


Figure 1.3 *Staged initiation at* oriC *in E. coli. I sites are not included in the figure (see text). The picture was obtained from Messer, 2002.*

Sequestration and SeqA

Sequestration is a mechanism in *E. coli* where newly replicated origins are prevented from re-initiating (Campbell and Kleckner, 1990). Dam methyl transferase, encoded by the *dam* gene recognizes 5′-GATC-3′ sites in the DNA double-strand and adds a methyl group to the adenine within every GATC site. The methylation occurs on unmethylated GATC sites which are generated after replication. At the time of replication initiation the adenine residues on both strands are methylated. This situation changes after replication because newly synthesized DNA strands are unmethylated, and since the old strand remains methylated this generates hemimethylated DNA.

SeqA, a 21 kDa protein is non-essential to the cell. SeqA protein binds a minimum of two hemimethylated GATC sites and has a high affinity for fully methylated *ori*C (Brendler and Austin, 1999;Skarstad *et al.*, 2000;Slater *et al.*, 1995). SeqA is not bound to unmethylated *ori*C (Slater *et al.*, 1995). The binding of SeqA to newly replicated hemimethylated *ori*C contributes to the ensuring that initiation occurs only once per cell cycle and simultaneously at all origins present in the cell (Boye *et al.*, 2000). It is assumed that SeqA binding to hemi-methylated origins is necessary for sequestration (Lu *et al.*, 1994). SeqA seems to have a role in folding of the chromosome (Fossum *et al.*, 2003b) and most likely in chromosome segregation (Bach *et al.*, 2003). It is also suggested that SeqA stimulates the relaxation and decatenation of TopoIV (Kang *et al.*, 2003).

1.2.2 Elongation and Termination

Synthesis of DNA requires a coordination of several proteins to form a DNA replication fork.

The replication forks move bidirectionally along the chromosome until they end up on the opposite side in the region called *ter* (terminus). Here the replication forks meet and terminate (Kornberg and Baker, 1992).

1.3 Chromosome segregation

After the replication has terminated the chromosomes are catenated due to the DNA structure.
In order to segregate the sister chromosomes the topologically constrained DNA needs to be decatenated. Topoisomerases, a class of enzymes, change the topological state of the molecule by introducing a transient break in the phosphodiester backbone, passing the DNA strand(s) through one another and reseal the break.

1.3.1 Topoisomerases

DNA topoisomerases regulate the number of topological links between two DNA strands by catalyzing transient single- or double-strand breaks, crossing the strands through one another and resealing the breaks (figure 1.4). Topoisomerases are classified as type I enzymes and type II enzymes, and both types exist in both prokaryotes and eukaryotes organisms.

Type I topoisomerases (Topo I and Topo III) in *E. coli* introduces single-strand nicks.

Type II topoisomerases consists of DNA gyrase and Topo IV. Both enzymes are composed of two subunits and both are essential for cell survival. The subunits of DNA gyrase, which is only present in prokaryotes, are named GyraseA and GyraseB, and the corresponding and homological subunits of Topo IV are ParC and ParE (Kato *et al.*, 1990).
These subunits combine into heterotetrameric complexes, gyrase A_2B_2 and Topo IV $ParC_2E_2$, to form active enzymes. The GyrB subunit and the ParE subunit are functionally similar as both contain the ATPase domain. The GyrA subunit and the ParC subunit are also similar and contain the DNA binding domain. Even if DNA gyrase and Topo IV seem alike only DNA gyrase is able to negatively supercoil relaxed DNA (Peng and Marians, 1995). ParC will be further explained below.

1.4 ParC and Topo IV

The *parC* gene was identified, via a mutation in *E. coli* that resulted in a partitioning defect. The mutant cells showed abnormal chromosome segregation (Kato *et al.*, 1988), and on background of showing defects in partition the gene was named *parC.* Mutations in *parC* or *parE* lead to the par phenotype. This phenotype is characterized by chromosomes that can be replicated but not partitioned. The result is accumulation of large nucleoids in the middle of cells at the non-permissive temperature.

ParC protein is composed of 752 amino acids (2259 bp) and has a molecular weight of 83 kDa. The native form of ParC is a homodimer and for ParE is a monomer. Topo IV may thus assemble in a stepwise fashion, *i.e.* $ParC_2$ + ParE → $ParC_2$ParE and $ParC_2$ParE + ParE → $ParC_2ParE_2$ (Peng and Marians, 1993b).

ParC produces transient double-strand breaks and removes supercoils two at a time. ParC is organized with the N-terminal domain that covalently attaches to the DNA strand which has a double-strand break (DNA gate). It is through this DNA gate that the T-segment must pass in order to change the superhelical density of the DNA substrate or decatenate sister molecules. In ParE the ATPase active site is at the N-terminal domain. The N-gate (figure 1.4) closes upon ATP binding and traps the T-segment on the DNA. ParE´s C-terminal domain is involved in the interaction with ParC.

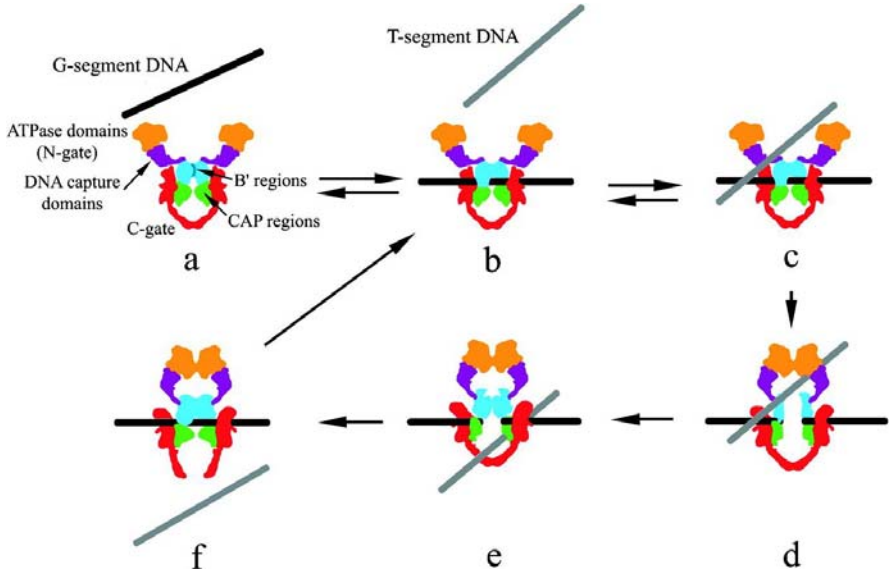


Figure 1.4 *Topoisomerase IV is a tetramer, and consists of 2 subunits ParE with a ATPase domain, here open and ready to capture the double-stranded DNA (T-segment). The T-segment will be transported through the $ParC_2$ which has catalyzed a DNA break in the G-segment. After the strand in transported through the break ParC religates the break. The picture was obtained from I. Flåtten, Dept of Cell Biology, R-R HF*

1.4.1 ParC´s function in the cell

In bacteria, successful cell division requires that the daughter chromosomes be disengaged topologically (decatenation), by the action of a topoisomerase, and that the daughter nucleoids be separated topographically (partition), one migrating to each of the daughter cells (Peng and Marians, 1993a;Peng and Marians, 1993b).

Topoisomerase IV primarily decatenates DNA and relaxes positive supercoils where the circular chromosome are catenated, or linked, and that this occurs prior to the completion of DNA replication (figure 1.5) (Adams *et al.*, 1992;Peng and Marians, 1993a;Peng and Marians, 1993b).

The decatenating activity happens as Topo IV catalyzes the ATP dependent passage of one DNA segment (the transport, or T-segment) through another segment (the gate, or G-

segment) (figure 1.4) and reseals the break. Topo IV Superhelical DNA relaxation requires both ParC and ParE (Kato *et al.*, 1992).

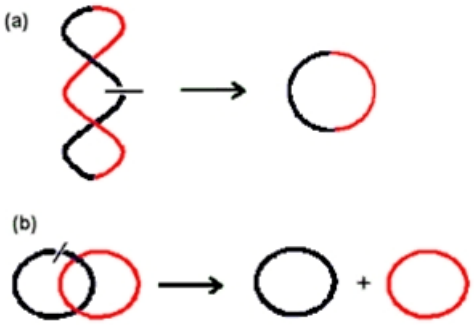


Figure 1.5 *a) Relaxing action of Topo IV, which involves a double-strand break (indicated by a short line), allowing the tangeled segment to pass through. The break is then resealed. b) The decatenation, where Topo IV makes a double-strand break in one DNA molecule (the black one), allowing the other molecule to pass through. The break is then resealed. The picture was obtained from I. Flåtten, Dept of Cell Biology, R-R HF*

1.4.2 ParC´s localization in the cell

It has been proposed that ParC localizes to the replication machinery and observations have linked Topo IV to the DNA polymerase III holoenzyme (HE) as well as other proteins (se below). Examination by immunostaining followed by fluorescence microscopy have localized ParC as discrete foci both between the nucleoid masses and on the DNA (Espeli *et al.*, 2003).

ParE seems to be appearing at the poles and in the center of the cell (Espeli *et al.*, 2003). This can indicate that Topo IV activity is regulated by separate localization of the subunits and that active Topo IV is suppressed until late in the cell cycle. It is not until ParC is disassembled from the replication factory that it is free to interact with ParE. The activity is restricted until the two daughter chromosomes are segregated (Espeli *et al.*, 2003). ParC localization in the cell are however uncertain and one of the aims of this thesis.

1.4.3 Interaction with SeqA

SeqA protein which prevents overinitiation of chromosome replication has been suggested to also participate in the segregation of chromosomes (Kang *et al.*, 2003).

It is shown that SeqA interacts with the C-terminal part of ParC and that SeqA stimulates Topo IV activity both in relaxing supercoiled plasmid DNA and converting catenanes to monomers. Earlier microscopy of immunolabeled SeqA has shown that SeqA is predominantly localized in foci situated at the replication forks. Here SeqA binds to newly replicated DNA and might contribute to proper segregation of daughter chromosomes (Bach *et al.*, 2003;Fossum *et al.*, 2003a;Fossum *et al.*, 2003b). Excess SeqA protein delays nucleoid segregation and cell division (Bach *et al.*, 2003).

1.4.4 Interaction with other proteins

Another identified protein possibly interacting with ParC is FtsK. It is shown that FtsK stimulates the decatenation activity of Topo IV (Espeli *et al.*, 2003). FtsK resides at the septum and aids in transportation of DNA to the correct cell halves. Espeli *et al.* proposed that FtsK contributed to concentrate ParC to the cell centre by the capture of ParC after the replication fork was disassembled.

1.5 Aims of the study

The localization of the ParC protein, its, if any, function at the replicationfork and the possible relationship with other proteins there, is not completely understood.
A previous investigation has shown that ParC colocalizes to the replication factory by interacting with DNA polymerase III holoenzyme and thereby forming discrete foci. Temporary evidence for this is reported by Espeli *et al*. I wanted to further confirm ParC protein's localization in *E. coli* cells.

The ParC protein has been found to interact with SeqA protein, where SeqA stimulates the activities of Topo IV, both in relaxing supercoiled plasmid DNA and decatenation of daughter chromosomes. In order to find out whether their localization in the cell were comparable I wanted to look at SeqA foci together with ParC foci.

The main objective of this study was to find out whether ParC colocalizes with SeqA at the replication factory.

2 METHODS

2.1 Growth conditions and storage of cells

6 ml LB medium was inoculated with a bacterial strain and 50 μg/ml ampicillin and 30 μg/ml chloramphenicol. The cells were grown for around 16 hours at 37°C. To ensure that the culture is adequately aerated, the volume of the culture should be at least four times greater than the volume of the bacterial culture. The tube should be loosely capped and the culture should be incubated with vigorous shaking. Overnight cultures are diluted 1:500 in fresh medium, containing the same amount of antibiotics and grown to exponentially phase at 37°C with vigorous shaking. To monitor growth mass optical density (OD) was measured at 450 nm for AB minimal medium or 600 nm for LB medium with a spectrophotometer.

2.2 Standard DNA techniques

2.2.1 DNA plasmid purification

(Sambrook and Russell, 2001)

Alkaline lysis in combination with the detergent SDS is used to isolate plasmid from *E. coli*. Exposure of bacterial suspensions to the strongly anionic detergent at high pH opens cell wall, denatures chromosomal DNA and proteins, and releases plasmid DNA into the supernatant. The alkaline solution completely disrupts base pairing, but since the strands of closed circular plasmid are topologically intertwined they will not separate. When the pH is returned to neutral the two strands fall back into register as long as the intensity and duration of exposure to OH^- is not too great.

The denatured DNA, broken cell walls and bacterial proteins form large complexes that are coated with dodecyl sulfate. By replacing the sodium ions with potassium ions the complexes are efficiently precipitated from solution. After centrifugation native plasmid DNA can be recovered from the supernatant.

The plasmid DNA recovered from the lysate can be purified in many different ways and to different extend, according to the needs of the experiment. Small scale and large scale DNA plasmid purifications are described in appendix 2 and 3.

2.2.2 Purification of DNA from gel bands

(Sambrook and Russell, 2001)

PCR products are commonly purified to remove excess nucleotides and primers. In preparation for cloning the DNA fragments need to be purified. This membrane-based system allows recovery of isolated DNA fragments or PCR products.
The principle for purification of DNA from gel bands is based on the ability of DNA to bind to silica membranes in the presence of chaotropic salts. Guanidine isothiocyanate is a strong chaotropic agent because it contains a reductant to break protein disulphide bonds and potent cationic and anionic groups that form strong hydrogen bonds. Hydrogen bonding profoundly affects the secondary structure of polymers such as DNA, RNA, and proteins, as well as how water-soluble a molecule is. Under native conditions, nucleic acids are covered by a hydrate shell consisting of water molecules that maintain the solubility of DNA in aqueous solutions. With the addition of chaotropic ions to the nucleic acid, this relatively ordered structure of water molecules of the hydrate shell is destroyed. The chaotropic salts create a hydrophobic environment. Under these hydrophobic conditions, the silica membrane column is the most suitable binding partner for the nucleic acids. Proteins, metabolites, and other contaminants do not bind to the membrane and therefore are washed away during the subsequent washing steps. As a further feature of the chaotropic salts, the respective cations saturate the silica membrane with positive charges,

which still improves the binding of nucleic acids under hydrophobic conditions. The high concentration of salt also facilitates binding of the nucleic acids DNA and RNA to the silica membrane in the column. The protocols describing the reaction are listed in appendix 4.

2.2.3 Quantification of DNA

(Sambrook and Russell, 2001)

The Hoefer® DyNA Quant 200 Fluorometer and Hoechst 33258 fluorescent dye were used to quantify DNA preparations of unknown concentration. Calf thymus DNA was used as a double stranded DNA standard. Hoechst 33258 binds to the AT-rich region and with high specificity into the minor groove of double stranded DNA. Hoechst 33258 excites in the near UV (350 nm) and emits at 458 nm, and when it is bound to an unknown sample and a DNA standard the concentration of the sample can be estimated by comparing the intensities of fluorescence.

An assay solution containing 0.1 µg/ml Hoechst 33258 in 1 x TNE- buffer was mixed with 1/1000 volume DNA sample or 1/1000 volume DNA standard (100 µg/µl).

2.2.4 Agarose gel electrophoresis

(Sambrook and Russell, 2001)

Electrophoresis through agarose is used to separate, identify and purify DNA fragments. Agarose is a polysaccharide isolated from seaweed. It is composed of alternating residues of D- and L-galactose joined by α-(1→3) and β-(1→4) glycosidic linkages (figure 7.1)

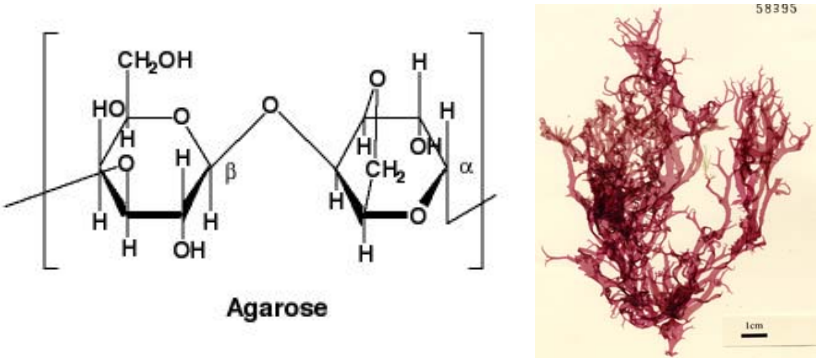


Figure 7.1 *Agarose consists of repeating monomers of 3, 6-anhydro-L-galactose and D-galactose. Agarose is a polysaccharide isolated from seaweed.*
The figures was obtained from www.bioc.unizh.ch/.../agar/agarose.jpg and agtcbioproducts.com/images/agarose01.jpg.

Gelation of agarose results in a three-dimensional mesh of channels whose diameters range from 50 nm to 200 nm. DNA from about 200 basepairs to approximately 50 kb in length can be separated on agarose gels of various concentrations (standard: 0.5-1.5 %)

Small DNA fragments are best resolved in agarose gels run in horizontal configurations, with an electric field of constant strength and direction.
The rate of migration of DNA through the gel depends upon several factors. Larger molecules migrate slowly because of greater frictional drag and because they worm their way through the gel less efficiently than smaller molecules. The type and concentration of agarose, the strength of applied voltage, the composition and ionic strength of electrophoresis buffer all affect the mobility of DNA. Superhelical circular and linear DNAs migrate through agarose gels at different rates. Under some conditions superhelical circular DNA migrates faster than linear DNA, under other conditions the order is reversed.

The DNA bands on agarose can only be visualized if they are labeled or stained in someway. One method is to expose DNA to the dye ethidium bromide (EtBr), which fluorescence under UV light when it is bound to DNA. Protocol is listed in appendix 5.

2.3 Cloning in E. coli

(Alberts *et al.*, 2002)

DNA cloning is a term, which in cell biology is used in two senses. In one sense it refers to the amplification of a DNA molecule. However, the term is also used to describe the isolating of one DNA sequence in order to create many identical copies of this particular gene. The cloning process can be accomplished in several ways. In this thesis a DNA fragment was inserted into a cloning vector, and the DNA propagated by insertion into it is said to have been cloned

2.3.1 The polymerase chain reaction (PCR)

(Alberts *et al.*, 2002)

PCR is an in vitro technique where selected regions of DNA are amplified a billion fold. Two sets of DNA primers (oligonucletides), chosen to flank the desired nucleotide sequence of the gene, are synthesized by chemical methods. In this thesis one of the primers, ParC F NcoI-His (table A1.2) contains a molecular tag of 6 amino acids histidine (His-tag). The His-tag, which is placed in N-terminus of the recombinant protein, is used in order to be able to purify the proteins using column chromatography (appendix 19). The two primers are then used to prime DNA synthesis on single strands generated by heating the DNA from the entire genome. The newly synthesized DNA is produced in a reaction catalyzed in vitro by a purified DNA polymerase, and the primers remain at the 5´ends of the final DNA fragments that are made.
Nothing special is produced in the first cycle of DNA synthesis; the power of the PCR method is revealed only after repeated rounds of DNA synthesis. Every cycle doubles the amount of DNA synthesized in the previous cycle. Because each cycle requires a brief heat treatment to separate the two strands of the template double helix, the technique requires the use of a special DNA polymerase. Taq polymerase, isolated from the thermophilic bacterium Thermus aquaticus, catalyzes template-dependant synthesis of DNA and is the

enzyme most frequently used. A version of Taq, AmpliTaq Gold™ DNA polymerase, which is used in this thesis, requires heat activation to generate polymerase activity. Taq polymerase is stable at a much higher temperature than normal, so that it is not denatured by the repeated heat treatments. With each round of DNA synthesis, the newly generated fragments serve as templates in their turn, and within a few cycles the predominant product is a single species of DNA fragment whose length corresponds to the distance between the two original primers.

The DNA oligonucletides to prime DNA synthesis requires careful design to obtain the desires products in high yield. Other components are equimolar amounts of dATP, dTTp, dCTP and dGTP, free divalent cations - usually Mg^{2+}, buffer to maintain pH, monovalent cations – usually KCl and template DNA. Template DNA containing target sequences can be added to PCR in single- or double-stranded form.

PCR is an iterative process consisting of 3 elements. First the DNA template is denatured to separate the double-strand helix. The temperature is determined in part of their GC content. The higher the GC content, the higher the temperature required. Annealing temperature, an important parameter in PCR, is adjusted according to GC content. Stability of the duplex is a direct function of the number of triple-hydrogen-bounded GC base pairs it contains; the larger the mole fraction of GC pairs, the higher the temperature of melting. In thermal melting, unwinding of the chains begin in regions high in AT base pairs, and proceeds to regions of progressively higher GC content (Kornberg and Baker, 1992).The time is determined partly by the length of the molecule. Annealing of primers to single-stranded target sequence requires the correct temperature. To optimize the annealing conditions the thermal cycler can be programmed to touchdown PCR. This involves using progressively lower annealing temperatures in consecutive pairs of cycles but having identical conditions in all other respects. Extension of the annealed primers by a thermostable DNA polymerase is carried out at or near the optimal temperature for DNA synthesis. The number of cycles required for maximum yield of amplification product is generally 30 cycles. The reaction was carried out as described in appendix 6.

2.3.2 Digestion of DNA by restriction nucleases

(Alberts *et al.*, 2002)

Restriction nucleases are different enzymes made of different bacteria. They are used as protection against virus infection by digestion and degrading of viral DNA. These enzymes can be purified from the bacteria, cut the DNA double helix at certain sites specified by the local nucleotide sequence, and cleave double-stranded DNA molecules into fragments. Each nuclease recognizes a specific sequence of four to eight nucleotides in DNA. Different restriction nucleases have different sequence specificities, making it simple to create a DNA fragment of strictly defined size, including a particular gene.
The bacteria's own genome is protected from cleavage by methylation at an A or a C residue. The sequences in foreign DNA are generally not methylated and will be cleaved. Some restriction nucleases produce staggered cuts, which leave short single-stranded tails at the two ends of each fragment. These ends are called *cohesive ends* since they form complementary base pairs with ends of other fragments cut by the same restriction nuclease or another enzyme that creates the same *cohesive ends*. Other restriction nucleases produce *blunt ends* which mean there are no single-stranded tails at the end of the fragment.

Recombinant DNA molecules are produced by splicing together complementary fragments with the enzyme DNA ligase as described in paragraph 2.3.3.

For complete digestion, 1 unit of restriction nuclease is required to digest 1 μg of substrate DNA in 1 hour. Supercoiled plasmids generally require more than 1 unit enzyme per μg DNA to be cleaved completely. The reaction was carried out as described in appendix 7.

2.3.3 Ligation

(Alberts *et al.*, 2002;Sambrook and Russell, 2001)(*pGEM®-T Easy Vector Instructions for use of products, Promega*)

DNA ligase is an enzyme that creates covalent phosphodiester bonds between 3` hydroxyl end of one nucleotide with 5` phosphate end of another. For its catalytic activity the enzyme requires the presence of ATP and Mg^{2+}. Ligase can link *cohesive ends* and *blunt ends* as well as creating a final phosphodiester bond in single strand breaks to fully repair the DNA.

T4 DNA ligase is used in most experiments and is isolated from bacteriophage T4. The optimal enzyme temperature is a balance of melting temperature and annealing temperature of the DNA fragments being ligated.

The molar ratio of insert DNA fragment to plasmid vector should be ~ 2:1 in the ligation reaction. The ideal volume for the reaction is 20 µl and is carried out at 16ºC over night or for 1 hour at room temperature. In the ligation reaction using pGEM-T Easy Vector the ideal volume was 10 µl. The reactions are described in appendix 7 and 13.

2.3.4 Transformation

(Madigan *et al.*, 2002;Sambrook and Russell, 2001)

In order to see the genetic change the recombinant molecule needs to be incorporated into a recipient cell. Cells that are able to take up DNA molecules and be transformed are said to be competent. One way to induce competence to *E. coli* is to treat the cells with high concentrations of calcium ions which make chemically competent cells. How the cells take up the DNA is not exactly known. Another technique is electroporation, were the cells are exposed to pulsed electrical fields. The cells plasma membranes are partly disrupted to make them more permeable and the DNA enter the cells through the small transient pores

before resealing. This method is temperature-dependent and is best carried out at 0-4 °C. This method gives electrocompetent cells. Ultracompetent cells are chemically competent cells which apparently have higher transformation efficiency. Preparation of the different competent cells, the respectively transformation and the selection of transformants are described in appendix 8-12 and appendix 14.

2.3.5 Subcloning

(Sambrook and Russell, 2001)(*pGEM®-T Easy Vector Instructions for use of products, Promega*)

The pGEM®-T Easy Vector System are systems for subcloning of PCR products. The vector is prepared by cutting the pGEM®-T Easy Vector with EcoR V and adding a 3` terminal thymidine to both ends. These single 3`-T overhangs at the insertion site greatly improve the efficiency of ligation of a PCR product into the plasmid by preventing recirculization of the vector. Certain thermostable polymerases provide a compatible overhang by adding a single deoxyadenosine to the 3′- ends of the amplified fragments. pGEM®-T Easy Vector contain T7 RNA polymerase promoter flanking a multiple cloning region within the α-peptide coding region of the enzyme β-galactosidase. α-complementation occurs when two inactive fragments of *E. coli* β-galactosidase associate to form a functional enzyme. β-galactosidase is encoded by the *lacZ* gene and the enzyme cleaves the disaccharide lactose into the monosaccharides glucose and galactose. Neither plasmid-encoded fragments of β-galactosidase nor the host-encoded fragments of β-galactosidase are themselves active, but they can associate to form an enzymatically active protein. The *lac*$^+$ bacteria that result from α-complementation form blue colonies in the presence of the chromogenic substrate X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), because X-gal is converted by the enzyme into an insoluble dense blue compound. When *parC*-His is inserted into the pGEM®-T Easy Vector the transformed bacteria will appear as white colonies. This is because the *lacZ* gene is disrupted by

insertion so that no plasmid-encoded fragment of β-galactosidase can be formed to join the host-encoded fragment of β-galactosidase. The protocol is listed in appendix 13.

2.4 DNA sequencing and sequence alignment

(Alberts *et al.*, 2002)

DNA sequencing, which is originally based on Sanger's method of sequencing use the dideoxy method where *in vitro* DNA synthesis is performed in the presence of chain-terminating dideoxyribonucleoside triphosphates (ddNTP). This derivative of a normal ddNTP lacks the 3´ hydroxyl group that allows for strand extension in the 5´- 3´ direction. When purified DNA is synthesized *in vitro* in a mixture that contains single-stranded molecules of the DNA to be sequenced, DNA polymerase, a primer, the four dNTPs and a small amount ddNTP, incorporation of ddNTP will terminate the growing DNA chain. This is because the lacking 3´OH group will block the next nucleotide. The reaction mixture contains both normal dNTPs and ddNTP, which leads to a competition between chain extension and infrequent, but base-specific, termination.
Accordingly, this reaction mixture will eventually produce, at random, a set of DNA fragments of different lengths. The complete reaction is run on a polyacrylamide gel which separates the fragments differing by only one nucleotide. To facilitate separation, the chain-terminating nucleotides are each labeled with a different colored fluorescent dye, the reactions can be performed in the same tube and the products be separated in a single lane on the gel. A detector positioned near the bottom of the gel reads and records the color of the fluorescent label on each band as it passes through a laser beam. A computer then reads and stores the nucleotide sequence.

2.4.1 Sequence alignment

Alignment of the sequence of *parC*-His insert and 4 different primers was performed using GATC Biotech http://www.gatc-biotech.com/en/index.php and Blast 2 Sequences http://www.ncbi.nlm.nih.gov/BLAST/bl2seq/wblast2.cgi

2.5 SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and detection of proteins by immunoblot analysis

(Alberts *et al.*, 2002)

Proteins have, depending on the mixture of the amino acids they contain, a net positive or negative charge. When an electric field is applied to a solution of proteins, the protein migrates at a rate depending on its net charge, its size and shape. This technique is electrophoresis and is the basis for SDS-PAGE.

Polyacrylamide gels are composed of chains of polymerized acrylamide that are highly cross linked by *N,N´*-methylene-bis-acrylamide. The pore size of the gel can be adjusted, by the amount of acrylamide, to "fit" the protein molecule of interest. Ammonium persulphate (APS) is a strong radical initiator and catalyze the polymerization of acrylamide and bisacrylamide. TEMED (*N,N,N´,N´*-tetramethylethylenediamine) is used with APS to contribute to the same polymerization. In order to load the proteins onto the gel they must unfold into extended polypeptide chains, and be released from other proteins or lipid molecules. Sodium dodecyl sulfate (SDS), which is an anionic detergent, binds to the hydrophobic regions of the proteins and render them freely soluble in detergent solution. In addition, the reducing agent β-mercaptoethanol is added to break any *S-S* linkages in the proteins. Polypeptides of equal size bind the same amount of SDS, attain similar negative charge and migrate towards the positive electrode, through the gel, with equal rate. The polyacrylamide gel, which acts as a molecular sieve, retards the large molecules more than the small one. As a result, a mixture of proteins is fractionated into a series of discrete bands arranged in order of molecular weight, which can be identified with a marker. The protocols are listed in appendix 16 and 17.

In detection of proteins by immunoblot analysis, a semidry blotting was used to transfer the proteins from the gel to a polyvinylidene difluoride (PVDF) membrane. In order to block unspecific binding sites on the membrane, the membrane was soaked in low-fat dried milk in TBS-T. Then irrelevant proteins from the milk could bind to the unspecific binding sites. This eliminated some background signals so that the immunoblotting could be more sensitive. First the membrane was incubated with primary antibody that bound to the protein of interest (figure 7.3). Then the membrane was incubated with a secondary alkaline phosphatase-linked antibody specific for the primary antibody, followed by addition of ECF substrate. The alkaline phosphatase catalyses the conversion of ECF, by cleaving a phosphate group from the ECF substrate to yield a highly fluorescent product which fluoresces at 550-570 nm. Protocols are listed in appendix 18.

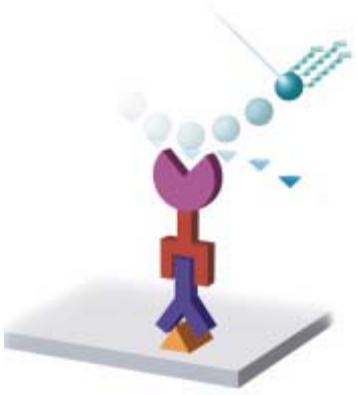


Figure 7.3 Schematic diagram of the ECF Western blot detection.
Orange symbol, target protein; purple symbol, primary antibody; red and pink symbol, secondary antibody with linked phosphatase; blue circles, ECF substrate; blue triangles, phosphate group. The picture was obtained from
http://www4.gelifesciences.com/applic/upp00738.nsf/vLookupDoc/193300141B500/$file/AppNote_68.pdf

2.5.1 Measurement of total protein content

(BCA Protein Assay Kit, PIERCE)

The Pierce BCA Protein Assay is based on bicinchoninic acid (BCA) for the colorimetric detection and quantification of total protein. This method combines the reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium with the binding of Cu^{1+} to BCA which results in the formation of a BCA-Cu^{1+} complex which exhibits a strong absorbance at 562 nm that is nearly linear with increasing protein concentrations over a broad range of 20 μg/ml to 2 000 μg/ml protein. The protocol is listed in appendix 16.

2.6 Expression of ParC protein

(Sambrook and Russell, 2001)

Expression of cloned genes is done to identify and characterize the recombinant protein they encode. The vector pET-16b contains a T7 promoter, and expression of a gene cloned into the T7 vector requires transformation into competent cells containing the T7 RNA polymerase gene, which BL21-Gold(DE3)pLysS does.

The plasmid vector pET-16b contains the *lacI* gene encoding the *lac* repressor. The *lac* repressor prevents transcription from the promoter in the absence of an inducer. When IPTG (isopropyl-β-D-thiogalactopyranoside) is added, the repressor will be unable to bind to the promoter and transcription of the gene starts accordingly.

IPTG (isopropyl-beta-D-thiogalactopyranoside) is a highly stable synthetic analog of lactose. It inactivates the *lac* repressor and induces synthesis of β-galactosidase, an enzyme that promotes lactose utilization. The IPTG is used to induce the expression of cloned genes which are under control of the *lac* operon. It is used in conjunction with X-Gal to determine the *lac* phenotype in blue/white colony screening.

X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside) is an inert chromogenic substrate for β-galactosidase. β-galactosidase hydrolyzes X-Gal into a colorless galactose and 4-chloro-3-brom-indigo, which forms an intense blue precipitate. Induction of the *lacZ* gene with IPTG leads to the hydrolysis of X-Gal and to the development of blue colonies. Protocol is listed in appendix 15.

2.6.1 Inclusion bodies

(Sambrook and Russell, 2001)

Most nonsecreted proteins synthesized in *E. coli* accumulate as denatured aggregates, called inclusion bodies. The formation of inclusion bodies is influenced by the nature of the protein, by the host cell and by the level of expression resulting from the vector choice and the growth and induction conditions. Inclusion bodies can be purified by differential centrifugation.
These aggregates are insoluble in aqueous buffers at neutral pH, but they usually can be dissolved in buffers that are markedly acidic or alkaline, or contain high concentrations of detergent, organic solvents or denaturants. In a few cases it has been possible to fold the solubilized protein in vitro into an active or native state. The protocol is described in appendix 19.

2.7 Purification of ParC protein

(Alberts *et al.*, 2002)

Proteins are most often fractionated by column chromatography. Since mixtures of proteins often are complex, several types of columns are usually employed to produce a highly purified protein fraction.

2.7.1 Affinity chromatography

(Ni-NTA His●Bind®Resins Instruction Manual, Novagen)

ParC protein was first purified by affinity chromatography. Affinity chromatography with the His●Tag technology is based on the binding between 6 (-10) histidines of the His●Tag sequence and an immobilized metal ion, Ni^{2+}-ions (or Cu^{2+}, Zn^{2+}). The histidine tag on the recombinant protein interacts with the metal ions and the protein is selectively retained on the column. The binding is reversible allowing for elution of the protein with buffers containing imidazol. Interaction between resin and his-tag of recombinant protein does not depend on tertiary structure and can therefore be purified under native or denaturing conditions. Protocols describing the affinity column preparations and purification are listed in appendix 19.

2.7.2 Fast Protein Liquid Chromatography

Gel-filtration chromatography, which separate proteins according to their size and also determine the protein size, was the second column used to purify ParC. Gel-filtration was preformed with Fast Protein Liquid Chromatography® System (FPLC). The gel filtration column (Superdex™ 200 10/300 GL) is formed of cross-linked Agarose matrix which attains a highly porous structure and therefore separates the molecules. The largest molecules are least withhold in the porous structure, and elution is therefore in order of decreasing size.

Molecules excluded from the pores of the stationary phase (100 % in the mobile phase) move down the column at the same speed as the mobile phase. They will leave the column after one void volume (V_o) of mobile phase has passed through the column. Molecules with partial access to the pores will be retarded in their respective degree of access to the pores,

and their elution volume (V_e) can be recorded. Molecules with full access to the pores will all move down the column at the same speed and remain unseparated from each other.

The molecular weight of molecules in the different elution fractions can be estimated from a calibration curve where the K_{av} values to a set of molecular weight standards have been plotted against the logarithm of their corresponding molecular weights.

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

The fractions containing the desired protein are pooled and dialyzed against an appropriate buffer. The protocol describing FPLC is listed in appendix 20.

2.8 Purification of ParC antibody

The purpose of purifying protein ParC was to make a ParC-column. Rabbit IgG to ParC was purified on the ParC column after isolation of ParC IgG on the Protein A Sepharose® CL-4B column.

A sample of ParC, earlier purified by our group, was injected into a rabbit at Rikshospitalet and the antiserum was later collected. Immunoglobulins were first isolated from rest of the rabbit antiserum using the immunosorbent Protein A Sepharose® CL-4B. Protein A Sepharose is prepared by covalently coupling Protein A to 4 % cross-linked agarose beads. Protein A binds to the Fc region (see Figure 7.2) of IgG through interactions with the heavy chain. Since only the Fc region is involved in binding, the Fab region (see figure 7.2) is available for binding antigen. IgG bound to Protein A was eluted with an acidic glycine buffer and the fractions with high ParC IgG content were coupled to a CNBr column.

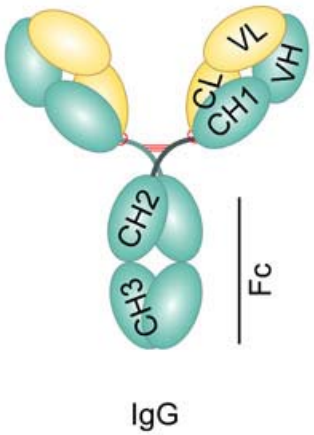


Figure 7.2 *Immunoglobulin G, built of two heavy chains (blue) and two light chains (yellow), Fc region, which binds to the protein A sepharose and Fab, the binding site for antigen. The picture was obtained from:* http://www.aapsj.org/articles/aapsj0802/aapsj080243/aapsj080243_figure1.jpg

To make the ParC column ParC protein was covalently coupled to CNBr activated sepharose in a column. Proteins containing primary amino groups can be coupled directly to the matrix. The antibodies specific to the antigen ParC, obtained from the protein A column, bind to the CNBr column, unreacted sites are blocked with glycin while the unbound antibodies and other serum proteins pass through the column. ParC IgG are then eluted from the column. Protocols describing preparation of columns and purification are listed in appendix 21 and 22.

2.9 In vivo characterization of ParC protein

2.9.1 Immunostaining and visualization of stained cells

(Alberts *et al.*, 2002;Sambrook and Russell, 2001)

Fluorescence microscopy is most often used to detect specific proteins in cells. A widely used technique is to couple fluorescent dyes to antibody molecules which then bind specifically to antibodies specific for the macromolecule of interest (figure 7.3)

The photons emitted by a dye are of lower energy (longer wavelength) than the photon absorbed. The illuminating light is passed through two sets of filters. The first filter lets through light with a narrow range of wavelengths that excite the particular fluorescent dye, while the second filter blocks out this light and passes only those wavelengths emitted when the dye fluoresces.

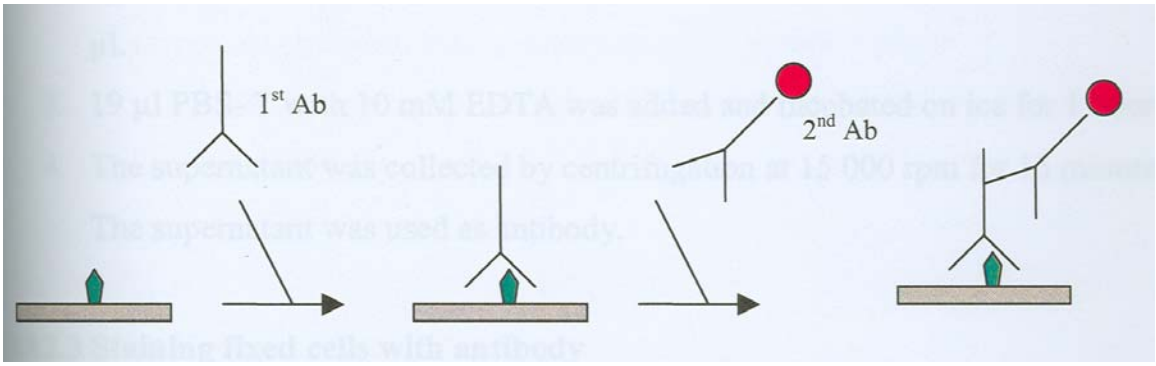


Figure 7.3 *Fluorescent labeling of a macromolecule by antibodies. Green symbol is macromolecule of interest, red symbol is fluorescent dye, 1st Ab is primary antibody specific for macromolecule and 2st Ab is secondary antibody specific for primary antibody. The picture was obtained from Ingvild Odsbus undergraduate thesis, 2003*

When looking at ParC foci, primary rabbit ParC antibody will bind to ParC in the cell. Anti-rabbit IgG labeled with fluorescent sulfoindocyanine dye, Cy3 is added and will bind to the primary antibody and give red fluorescence when viewed with the correct filter.

For viewing SeqA foci, the primary anti-SeqA antibody is first purified by incubation with cell extract from EBO193 (Δ *seqA*) that will attract unspecific antibodies, so that only specific anti-SeqA antibodies are left in the supernatant collected after centrifugation. The supernatant is used as antibody and secondary antibody labeled as described for ParC. DNA is labeled with Hoechst 33258 that emits blue light (appendix 24)

2.9.2 Visualization of immunostained cells

Visualization of immunostained cells was performed using a Zeiss Axioplan2 phasecontrast/fluorescence microscope equipped with a 63 x objective and a BP546/12 excitation filter. DNA was visualized with a BP365/12 excitation filter. Pictures were taken using a MicroMax CCD camera (Princeton Instruments) that was connected to a computerized image analysis system (Axiovision2 Multichannel, Zeiss). The optical images, which showed cell outlines, were merged with the fluorescent images, which showed fluorescent ParC and SeqA proteins.

3 RESULTS

3.1 Construction of new E. coli ParC overproducer strain

In order to develop a plentiful source for purification, *E. coli* stain overexpressing ParC via T7 RNA polymerase expression system was constructed. The *parC* gene was first subcloned into the plasmid pGEM-T Easy Vector and positive clones were identified by α-complementation (paragraph 2.3.5). Then the gene was cut out and cloned into the expression vector pET-16b behind a T7 promoter that was transformed into a competent *E. coli* strain BL21-Gold(DE3)pLysS. In this strain T7 RNA polymerase is expressed after induction with IPTG.

3.1.1 Cloning of *parC* in *E. coli*

Amplification of the gene *parC* by PCR was tested with the wild type stains MG1655 and W3110 as template. This gave no result, but by using the plasmid pIF01 containing *parC* and a primer with his-tag (see below), amplification was carried out.

It was first attempted to clone *parC* into pET-16b without subcloning.

The *parC* gene was amplified using the primers ParC F NcoI-His and ParC R BamHI (Table A1.3). The *parC* gene with his-tag and the ParC protein with his-tag will be referred to as *parC*-His and ParC respectively. Both primers contained specific restriction sites (underlined, see table A1.3) in the 5`termini that were copied to the termini of the complementary DNA sequences. The forward primer; ParC F NcoI contained the restriction site NcoI, and the reverse primer, ParC R BamHI contained the BamHI restriction site. The amplified DNA fragments were cleaved with the correct restriction enzymes (appendix 7) to make DNA fragments with *cohesive ends*.

The expression vector pET-16b was purified from DH5α *E. coli* cells by small scale DNA plasmid purification (appendix 2) and cut with BamHI and NcoI (appendix 7). Both restriction sites are situated in the pET-16b cloning region.

After digestion the DNA fragment the plasmid were purified from agarose gel bands as described in appendix 5.

A ligation mixture of *parC*-His and pET-16b was prepared with a molar ratio of insert to vector of 2:1 (appendix 7). Electrocompetent cells were prepared from DH5α *E. coli* cells (appendix 8) but electroporation (appendix 9) gave no colonies when the cells were diluted and spread on ampicillin containing LB plates. Further, chemically competent cells from DH5α *E. coli* cells, made with $CaCl_2$, were prepared (appendix 10). Transformation of recombinant plasmid into the cells (appendix 10) gave negative results.

A comparison of 3 different types of competent cells was carried through (table 3.1). Seven different transformation reactions were performed for both electrocompetent and $CaCl_2$-competent cells in addition to 2 ligation reactions into Ultracompetent XL10-Gold *E. coli* cells (appendix 11). The first reaction was a control for ampicillin resistance to see if the respective cells were able to grow on the plates by themselves. The next four reactions were controls where the respective competent cells were transformed with supercoiled plasmid in four concentrations to see if the transformation process worked satisfactorily. The final two reactions were transformations with the plasmid possibly containing the correct insert, in two different concentrations.

Table 3.1 *Results from comparing transformation of vector pET-16b with parC insert into electrocompetent, $CaCl_2$ competent and ultracompetent E. coli cells*

Reaction	Transformation reaction	Transformants
Electrocompetent cells:		
1. Control: ampicillin resistance	Competent DH5α cells	0
2. Control: transformation	Competent DH5α cells + supercoiled pET16b 100 µl	not countable
3. Control: transformation	Competent DH5α cells + supercoiled pET16b 10 µl	not countable
4. Control: transformation	Competent DH5α cells + supercoiled pET16b 1 µl	1060
5. Control: transformation	Competent DH5α cells + supercoiled pET16b 0.1 µl	177
6. Ligation	Competent DH5α cells + pET16b with parC-His 10 µl	0
7. Ligation	Competent DH5α cells + pET16b with parC-His 100 µl	0
$CaCl_2$ competent cells:		
1. Control: ampicillin resistance	Competent DH5α cells	0
2. Control: transformation	Competent DH5α cells + supercoiled pET16b 100 µl	336
3. Control: transformation	Competent DH5α cells + supercoiled pET16b 10 µl	34
4. Control: transformation	Competent DH5α cells + supercoiled pET16b 1 µl	3
5. Control: transformation	Competent DH5α cells + supercoiled pET16b 0.1 µl	0
6. Ligation	Competent DH5α cells + pET16b with parC-His 200 µl	0
7. Ligation	Competent DH5α cells + pET16b with parC-His 800 µl	2
Ultracompetent cells:		
1. Ligation	Ultracompetent XL10-Gold cells + pET-16b with parC-His 200 µl	0
2. Ligation	Ultracompetent XL10-Gold cells + pET-16b with parC-His 800 µl	0

The electrocompetent and the chemically competent cells gave 10^6 colonies per µg DNA and 10^3 colonies per µg DNA respectively. Ultracompetent cells should, according to producer give 10^7 colonies per µg DNA. The two positive transformants from reaction 7 into $CaCl_2$ competent cells were selected (appendix 12).

The possibly recombinant plasmids were purified by small scale DNA plasmid purification (appendix 2), digested with the appropriate restriction enzymes (appendix 7), prior to

identifying the sizes of the DNA fragments on an agarose gel (appendix 5). The vector was found without insert.

Instead of further attempts with almost identical conditions it was decided to try subcloning (appendix 13).

The ligation reactions, in two concentrations, of *parC*-His and pGEM®-T Easy Vector (paragraph 2.3.5) were done. The following transformations of recombinant plasmid into JM109 High Efficiency Competent Cells, (table 3.2) were performed as described in appendix 15. Insertional inactivation of the α-peptide allows recombinant clones to be directly identified by color screening on indicator plates (paragraph 2.6) At this stage of cloning the control reactions were leaved out since the procedures were known.

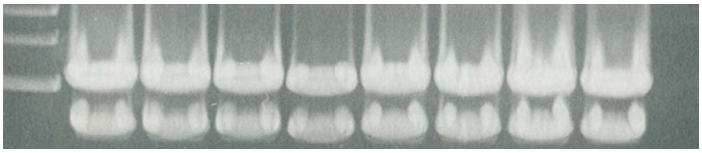
Table 3.2 *Results from transformation of pGEM-T Easy Vector with parC insert, into JM109 High Efficiency Competent E. coli Cells.*

Reaction	**Transformation reaction**	**Transformants**
1. Ligation	JM109 + pGEM-T with parC-His 100 µl	1 white, 6 blue
2. Ligation	JM109 + pGEM-T with parC-His 900 µl	49 white, 41 blue

Positive transformants (white colonies) from both reactions were selected (appendix 12)

To check if the correct fragments had been inserted, the recombinant plasmids were purified by small scale DNA plasmid purification (appendix 2) and digested (appendix 7). Finally the fragments were identified on a 0.8 % agarose gel where the linearized subcloningvector and the *parC*-His insert constituted 3015 and 2259 base pairs, respectively (figure not shown).
One plasmid construct (# 2.6) was chosen, and identified on a 0.8 % agarose gel a second time at 80 V for 2 hours for optimal separation (figure 3.1).

1 ---------- 2 ----------



← 3015 bp
← 2259 bp

Figure 3.1 *Digestion of the pGEM-T Easy Vector with possible parC-His insert*
Lane 1, 2-Log DNA ladder, lanes 2, purified plasmid from positive transformant digested with BamHI and NcoI, possibly containing the parC-His insert.

The plasmid was named pEE1 (figure 3.2).

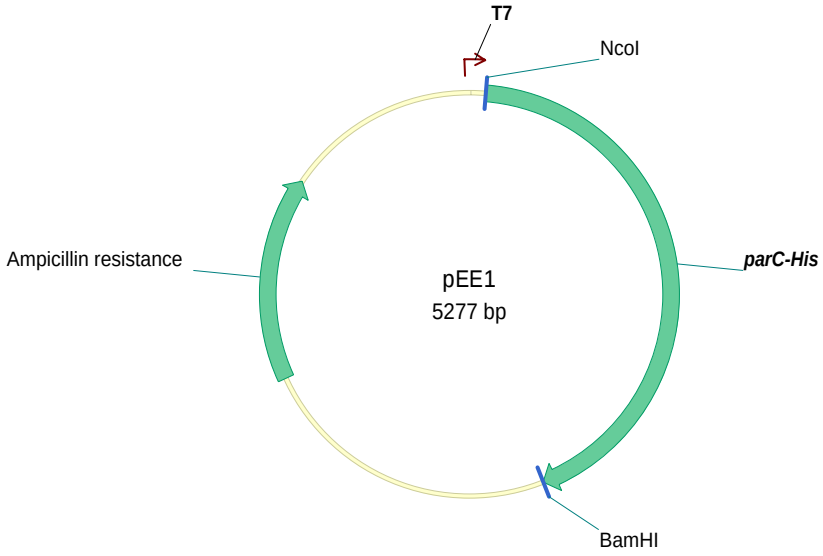


Figure 3.2 *Map of plasmid pEE1 with the insert parC-His inserted into pGEM-T Easy Vector. The plasmid is 5277 bp in size and in addition to the parC-His gene the ampicillin gene, the T7 promoter and the restriction sites for BamHI and NcoI are indicated.*

DNA sequencing was preformed to confirm that the correct fragment had been inserted into the plasmid pEE1 during cloning (paragraph 2.4).

In order to create the new ParC overproducer strain, the plasmid pEE1 and the expression vector pET-16b was purified by large scale purification (appendix 3). The insert *parC*-His was cut out of pEE1 with BamHI and NcoI and ligated into the vector pET-16b which had earlier been cut with the same enzymes. The new construct was transformed into Ultracompetent XL10-Gold *E. coli* cells (paragraph 11) and the transformants were selected (appendix 12) on LB plates containing 50 µg/ml ampicillin (table 3.3). The control reactions were leaved out due to familiarity of the system.

Table 3.3 *Results from transformation of vector pET-16b with parC insert into ultracompetent XL10-Gold E. coli cells.*

Reaction	**Transformation reaction**	**Transformants**
1. Ligation	Competent XL10-Gold cells + pET 16b with *parC*-His 100 µl	130
2. Ligation	Competent XL10-Gold cells + pET 16b with *parC*-His 900 µl	848

Positive transformants from both reactions were selected (appendix 12).

To verify the correct insert, the recombinant plasmids were purified by small scale plasmid purification (appendix 2) and digested with BamHI and NcoI (appendix 7). The fragment was identified on an agarose gel where the linearized vector and *parC*-His insert constituted 5711 and 2259 base pairs, respectively (figure 3.3).

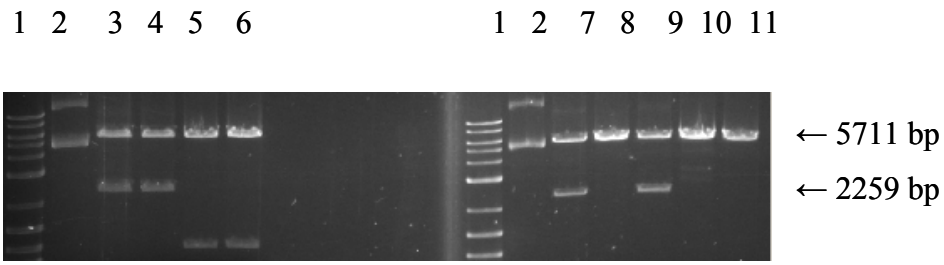


Figure 3.3 *Digestion of the vector pET-16b with possible parC-His insert.*
Lanes 1, 2-Log DNA ladder, lanes 2, linearized pET16b vector (digested with BamHI and NcoI), lanes 3-11, purified plasmid from positive transformants digested with BamHI and NcoI possibly containing the parC-His insert.

The plasmid construct in lane 7 was chosen and the plasmid was named pEE2 (figure 3.4).

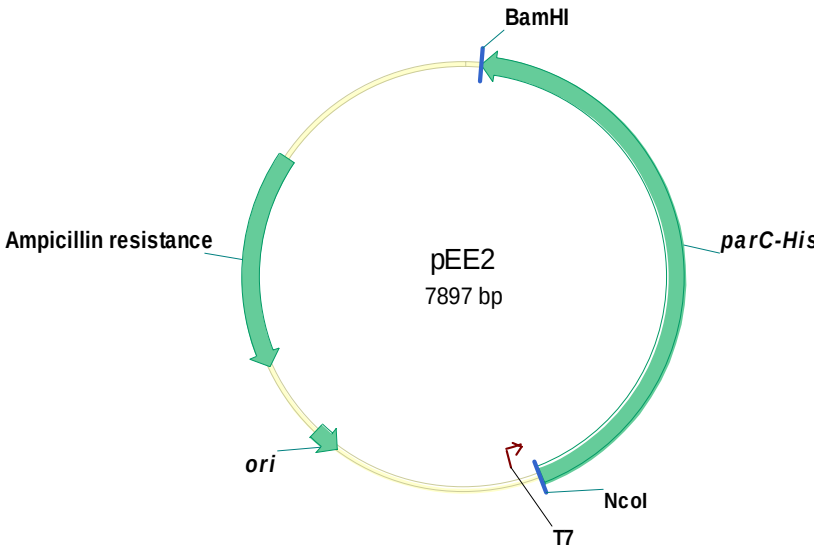


Figure 3.4 *Map of plasmid pEE2 with the insert parC-His inserted into the vector pET-16b. The plasmid is 7897 basepairs in size and beside the parC-His gene the ampicillin gene, the origin, the T7 promoter and the restriction sites for BamHI and NcoI are indicated.*

The plasmid pEE2 was purified by large scale purification (appendix 3) for further experiments.

3.1.2 DNA sequencing and sequence alignment

DNA sequencing was performed twice during the cloning process to verify that the recombinant plasmids had the right insert. Each reaction sequenced only 700 basepairs, so *parC* with 2259 basepairs needed four parallels of recombinant plasmid with one primer for each parallel to sequence the whole insert.

To amplify *parC* the following primers were used:

- *parC* F NcoI-His, from basepair 1-620.
- *parC* + 542, from basepair 605-1329.
- *parC* + 1192, from basepair 1253-1977
- *parC* R BamHI, from basepair 1595-2259.

The sequences of the *parC* insert in both recombinant plasmids were compared to the *parC* gene amplified by using the above-mentioned primers by pairwise alignment (paragraph 2.4.1). Neither pEE1 nor pEE2 had mutations so the cloning had been successful.

3.2 Expression of ParC protein

The plasmid pEE2 was transformed into BL21-Gold(DE3)pLysS competent *E. coli* cells (appendix 14) and the transformants were selected (appendix 12) and grown overnight in LB medium, 50 µg/ml ampicillin and 30 µg/ml chloramphenicol.

3.2.1 Finding conditions for optimal expression of ParC

It was important to find the optimal expression of ParC protein to get a high benefit from the purification. Overnight cultures from transformants of BL21-Gold(DE3)pLysSpEE2 were diluted 1:10 in LB medium and grown with vigorous shaking at 37ºC for 2 hours. Then 0.6 mM or 1 mM IPTG was added to the cultures and the cells were allowed to grow

for another 2 ½ hours. Samples for identification by SDS-PAGE (appendix 16 and 17) were collected before and after addition of IPTG (figure 3.5 and 3.6).

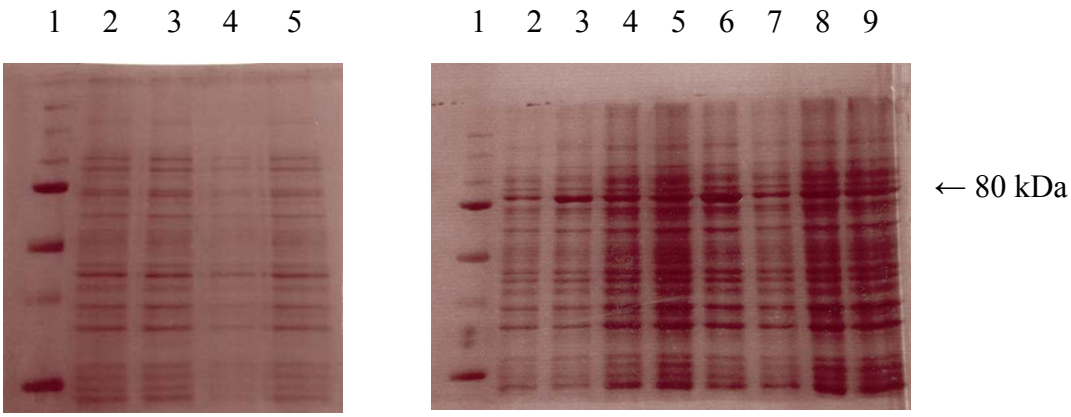


Figure 3.5 *Samples of uninduced (IPTG) ParC.*
Lane 1, Prescision Plus Protein® Dual Color Standards (marker), lane 2, BL21-Gold(DE3)pLysSpEE2#5, lane 3, BL21-Gold(DE3)pLysSpEE2#6, lane 4, BL21-Gold(DE3)pLysSpEE2#7, lane 5, BL21-Gold(DE3)pLysSpEE2#8.

Figure 3.6 *Samples of induced (IPTG) ParC.*
Lane 1, Prescision Plus Protein® Dual Color Standards (marker), lane 2, BL21-Gold(DE3)pLysSpEE2#5 induced with 0.6mM IPTG, lane 3, BL21-Gold(DE)pLysSpEE2#5 induced with 1 mM IPTG, lane 4, BL21-Gold(DE3)pLysSpEE2#6 induced with 0.6 mM IPTG, lane 5, BL21-Gold(DE)pLysSpEE2#6 induced with 1 mM IPTG, lane 6, BL21-Gold(DE)pLysSpEE2#7 induced with 0.6 mM IPTG, lane 7, BL21-Gold(DE)pLysSpEE2#7 induced with 1 mM IPTG, lane 8, BL21-Gold(DE)pLysSpEE2#8 induced with 0.6 mM IPTG, lane 9, BL21-Gold(DE)pLysSpEE2#8 induced with 1 mM IPTG

The clone and IPTG concentration (appendix 15) which gave the optimal expression of protein ParC, was BL21-Gold(DE)pLysSpEE#7 which was induced with 0.6 mM IPTG. The strain BL21-Gold(DE)pLysSpEE2#7 will be referred to as EE3.

The protein appeared at 80 kDa.

To further analyze the protein expression the duration of the IPTG induction time was tested. EE3 was induced with 0.6 mM IPTG (appendix 15) for 1, 2 and 3 hours. Samples were taken and identified by SDS-PAGE (appendix 16 and 17) (figure 3.7).

1 2 3 4

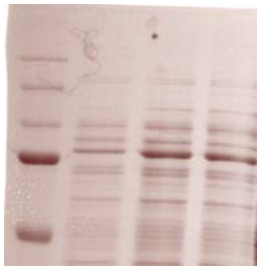


Figure 3.7 *Durations of induced ParC expression.*
Lane 1, Prescision Plus Protein® Dual Color Standards (marker), lane 2, EE3 induced with 0.6 mM IPTG for 1 hour, lane 3, EE3 induced with 0.6 mM IPTG for 2 hours, lane 4, EE3 induced with 0.6 mM IPTG for 3 hours.

The optimal duration of induction was 2 hours, which was chosen.

3.2.2 Solubilization of ParC in the extract

Many proteins are poorly soluble when overproduced and accumulate into inclusion bodies (paragraph 2.6.1). To compare the quantity of ParC in the supernatant and the pellet the following test was done; an overnight culture IPTG was centrifuged at 16 000 rpm for 1 minute, and the supernatant was removed. The pellet was resuspended in 1 ml LB medium, 20 µl BugBuster, 2 µl Benzonase was added and the samples were identified on a SDS- PAGE (appendix 17) (Figure 3.8).

1 2 3 4 5 6 7

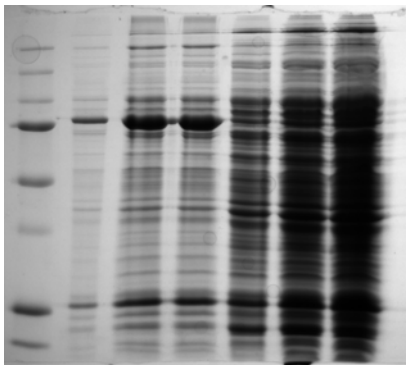


Figure 3.8 *ParC protein distributions in inclusion bodies and supernatant. Lane 1, Prescision Plus Protein® Dual Color Standards (marker), lane 2, 10 µl solution with ParC protein from pellet, lane 3, 40 µl solution with ParC protein from pellet, lane 4, 50 µl solution with ParC protein from pellet, lane 5, 2 µl solution with ParC protein from supernatant, lane 6, 5 µl solution with ParC protein from supernatant, lane 7, 10 µl solution with ParC protein from supernatant.*

Inclusion body purification was preformed as described in appendix 19. Despite the test that verified a higher content of ParC protein in the pellet, and one theoretically should believe ParC was easily solubilized, the method was not possible to accomplish in reality and therefore left out.

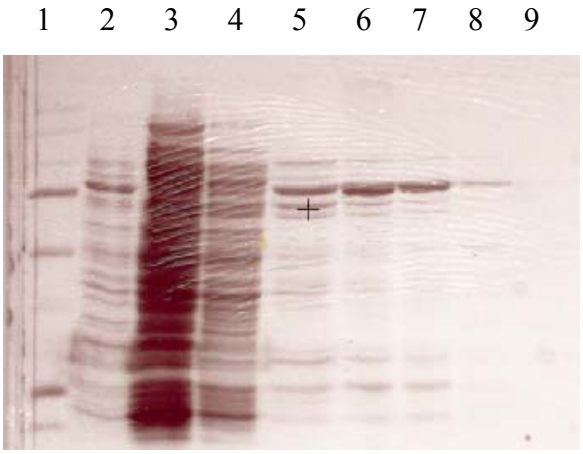
3.3 Purification of ParC protein

Purification of ParC protein was done by affinity chromatography and fast protein liquid chromatography (FPLC).

Six histidine residues were added to the *parC* gene during amplification (paragraph 2.3.1) which gave rise to a recombinant protein with a His-tag in the N terminal end. This His-tag aided the purification process by selective binding to Ni^{2+} ions that had been charged onto to column as described in appendix 19.

3.3.1 Purify protein from supernatant on Ni^{2+}Column

The lysate-Ni-NTA His-Bind slurry mix was loaded onto the column and the purification was preformed with step elution where the imidazole concentration increased in steps from 10 mM, 20 mM to 40 mM imidazole. Elution was at 250 mM (appendix 19). The fractions were identified on SDS-Polyacrylamide gel (figure 3.9).



Figur 3.9 *Purification of ParC protein on Ni^{2+}Column*
Lane 1, Prescision Plus Protein® Dual Color Standards (marker), lane 2, load - EE3, induced with IPTG 0.6 mM for2 hour, lane 3, flow-through during application of sample onto the column, lane 4, wash solution A (20 mM imidazol), lane 5, wash solution B (40 mM imidazol), lanes 6-9, elution fractions 1-4 (250 mM imidazol).

The eluation fractions 1 and 2 from the Ni^{2+}- column were pooled and concentrated on a Centricon centrifugal filter device.

3.3.2 Purification of ParC on FPLC

The pooled and concentrated fraction from the nickel column was loaded onto the gel filtration column (Superdex 200 10/300 GL) and fast protein liquid chromatography was preformed (appendix 20). The protein showed peak elution (elution of protein within a few fractions).The peaks were registered and fractions containing the possibly correct protein were chosen after estimating their size as explained in paragraph 2.7.2. The estimated size in fraction 2 and 9 (figure 3.10) were ~160 kDa which could be explained either from possible aggregation of ParC proteins or that ParC was bound to another protein. The column was loaded twice due to size of the loop (500 µl), so fractions 2 and 9 both have proteins of approximately equal size. The fractions containing the possibly correct protein were identified on a SDS-polyacrylamide gel (figure 3.10).

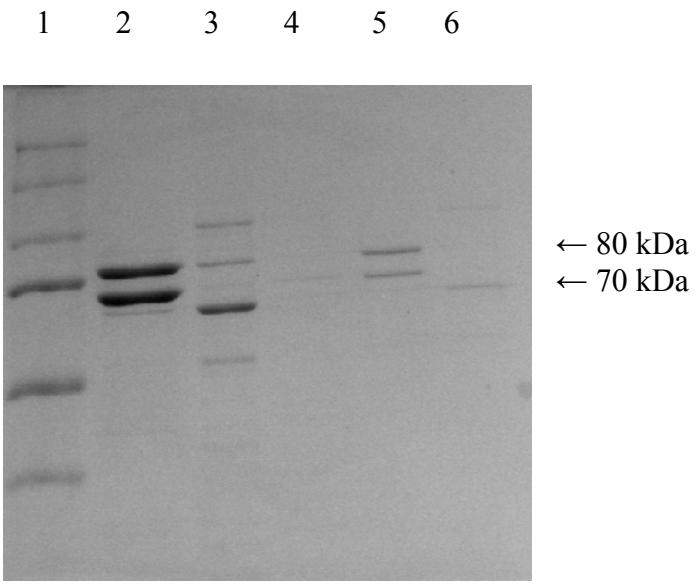


Figure 3.10 *Purification of ParC protein on FPLC. Lane 1, Prescision Plus Protein® Dual Color Standards (marker), lane 2, fraction 2; lane 3, fraction 3; lane 4; fraction 8; lane 5, fraction 9; lane 6, fraction 10*

The fractions in lane 2 and 5 (figure 3.10) were combined and dialyzed against 500 ml ice-cold dialysis buffer for 4 hours and then in 500 ml fresh dialysis buffer overnight.

3.4 Preparing the CNBr-ParC column

In order to remove additives present in the CNBr activated sepharose (paragraph 2.8) from producer, the resin was washed at low pH (2-3) (appendix 21) before coupling ParC ligand.

ParC protein, 2.5 mg was diluted in 10 ml coupling buffer and mixed with the washed and swelled CNBr resin (appendix 21). Unreacted ParC ligand, if any, is washed away and unreacted groups on the resin are blocked. To find out if the protein was completely coupled to the CNBr resin the protein concentration in the supernatant was measured. The supernatant contained no protein so ParC was almost completely bound to the column. The column was stored in 1 M NaCl/0.01% NaN_3 at 2-8°C.

3.5 Purification of ParC antibody

As for the CNBr activated sepharose also Protein A Sepharose CL-4B needed to be washed to remove additives. After washing and swelling the gel was packed onto the column.

ParC antiserum, obtained from Rikshospitalet after immunization of a rabbit (paragraph 2.8), was loaded on the protein A column (paragraph 2.8) (appendix 22), and samples from flow through, washing and elution were taken for identification on SDS-polyacrylamide gel (appendix 17).

Elution fractions with high IgG content from the protein A column were pooled (fractions 2-11) and mixed with CNBr-ParC resin. The IgG-CNBr-ParC resin was loaded on a

column, mixed for 2 hours and eluted with 10 fractions of 0.5 ml. The purified antibody was detected by immunoblot analysis (appendix 18). Cell extract for this was made, and included in SDS gel electrophoreses at 20 µg per lane (appendix 17). ParC antibody from elution faction 2 and elution fraction 3, both from the ParC column (with IgG-CNBr-ParC) were diluted 1:200. DnaA antibody, which was included as a positive control for Western, was diluted 1:10 000 (figure 3.11). Left membrane was incubated with DnaA antibody, middle and right membrane were incubated with ParC antiserum purified against both ProteinA column and CNBr-ParC column.

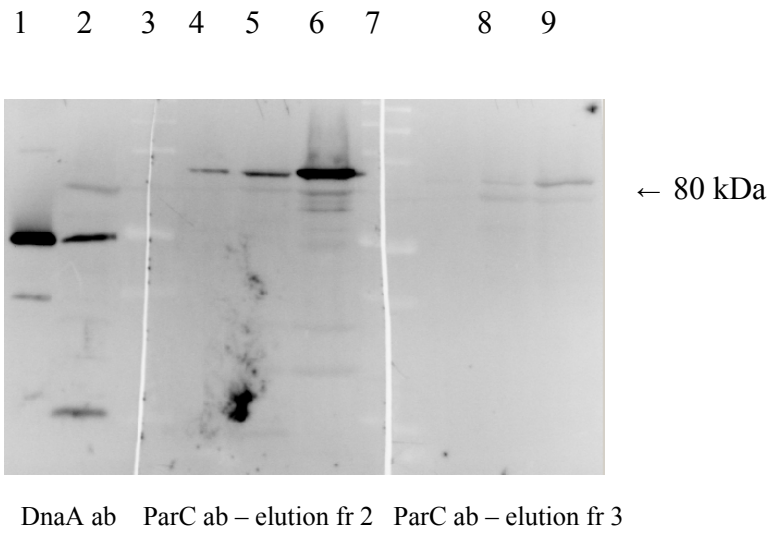


Figure 3.11 *Immunoblot of purified ParC antibody. Lane 1, DnaA protein 10 ng; W3110 wt 20 µg; lane 3 and 7, Prescision Plus Protein® Dual Color Standards (marker); lane 4, ParC 10 ng; lane 5, W3110 wt 20 µg; lane 6, EE3 (op) 20 µg; lane 8, ParC 10 ng; lane 9, W3110 wt 20 µg.*

After exposing the loaded samples to ParC antibody, and then exposing them to a secondary antibody (appendix 24) this is what was used for fluorescence microscopy (figure 3.11).

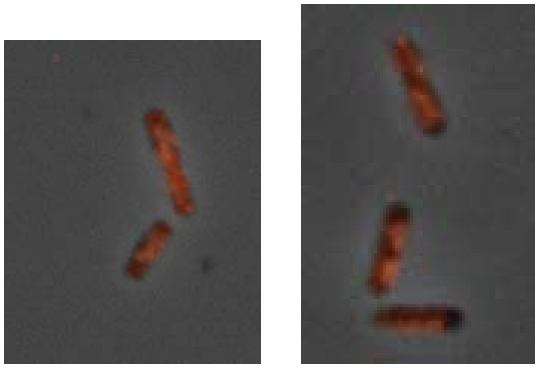
3.6 Immunostaining followed by fluorescence microscopy

It has earlier been proposed that ParC localizes as discrete foci on the DNA and between the nucleoid masses, and that there is an association between ParC and the replication machinery in the cell (Espeli *et al.*, 2003). In addition, earlier observations indicate that SeqA interacts with ParC (Kang *et al.*, 2003). I wanted to investigate whether these results were reproducible.

Cells from strains MG1655 were grown as described in appendix 16, fixed and stained as described in appendix 23. The cells were visualized as described in paragraph 2.9.2.

In W3110 wild type cells immunostained with SeqA, from one and up to 7 foci were observed but there were most of two or four fluorescent foci (figure 3.12 b), and in accordance with previous results (Molina and Skarstad, 2004). In W3110 wild type cells immunostained with ParC antibody discrete fluorescent foci were not observed, instead the fluorescence was fairly uniformly distributed (figure 3.12 a).

a)



b)

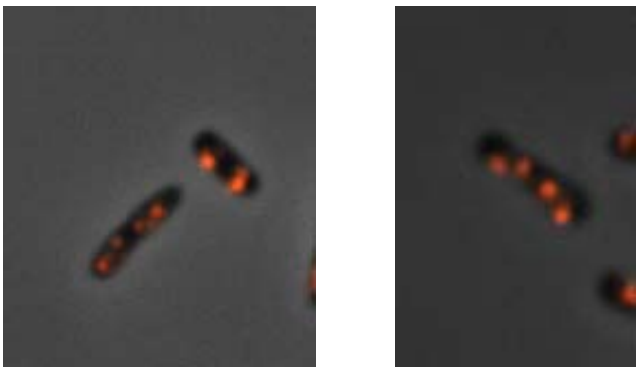


Figure 3.12 *Localization of immunostained ParC (a) and SeqA protein (b) in fixed wild type cells.*

4 DISCUSSION

4.1 Localization of the ParC protein

I find here that ParC antibody is not localized as discrete foci, but instead appears throughout the cell. This finding indicates that ParC protein does not colocalizes to the replication factory. However, this is a negative result and could therefore also be caused by a failure to detect intracellular ParC protein properly. A failure to detect ParC could be due either to problems with the visualization of ParC itself or with occurrence of signal not specific to ParC

There is during the process of my work, an indication that the ParC protein used in serum purification is not optimally purified. After running the FPLC gel filtration and identifying the samples on SDS-polyacrylamide gel (figure 3.10) there appears a second band just beneath ParC. This protein is about 70 kDa. Since Topo IV is a complex of two subunits each of the ParC and ParE polypeptides (Peng and Marians, 1993b) and since the active form of Topo IV is a heterotetramer, $ParC_2ParE_2$, this second band could be ParE. If so, ParE would also be present on the serum purification column and antibody against ParE would be present in the localization experiments. Par E has been found to be distributed uniformly throughout the cell (Espeli *et al.*, 2003), and could therefore explain the result. I consider this unlikely, however, because ParC was purified from a strain that overexpresses ParC. ParE is not overexpressed and exists only in its native quantity. It is thus not likely that the second band is ParE, and likewise unlikely that the cause for the uniform fluorescence distribution was detection of ParE.

Another explanation for the second band could be that some of the ParC protein had degraded during the purification process. This would give a smaller protein with fewer amino acids than ParC. This was tested by running another SDS-PAGE with the same

sample after two hours. The gel showed that the protein sizes and the mutual proportions were the same as previous.

4.2 Antibody entering the cell

As mentioned above, a problem with the visualization of protein could be due to failure of ParC antibody entering the cell during immunostaining.
Fixation and permeabilization of the cells is necessary for this to happen.
One major difference between my protocol and Espeli et al (Espeli *et al.*, 2003) was that I fixed the bacteria with 77 % ethanol while Espeli fixed with 2.4 % paraformalaldehyd and 0.01 % glutaraldehyd. The difference in methods could be a reason for the problem with the visualization of the protein.
This explanation is also unlikely since the same protocol was used when fixing and immunostaining with SeqA antibody.

4.3 Future plans

The next step in this study would be to vary the conditions for different methods. Due to spending much time with cloning there was little time left for this in the end of my study. The goal is to make the starting material contain more of what is overexpressed.
In addition, beside ParC´s possible localization to the replication machinery it wound be interesting to find out if ParC has any function there and what it involves. Are the subunits of Topo IV distributed in different subcellular locations?
The likely interaction between SeqA and ParC ought to be further investigated to find out more about ParC´s role at the replication factory.

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6 APPENDIX LIST

APPENDIX 1

Materials

Bacterial strains and plasmids

Table A1.1 *Bacterial strains used in this thesis*

Strain name	Relevant features	Plasmid	Source
BL21-Gold(DE3)pLysS	IPTG, *lacUV5*	-	Stratagene
DH5α	*rec*A1, *ø80 lac* ZΔM15	-	Laboratory stock
EE3	BL21-Gold(DE3)pLysS	pEE2	This work
JM109 Competent Cells	*rec*A1, *lac*I^{q}ZΔM15	-	Promega
MG1655	Wild type	-	Guyer *et al.* -81, Jensen -93
W3110	Wild type	-	Laboratory stock
XL10-Gold Ultracompetent cells	X-gal, IPTG, lacZ	-	Stratagene

Table A1.2 *Plasmids used in this thesis*

Plasmid	Relevant features	Selection	Source
pEE1	pGEM-T Easy Vector *parC*-His	Amp	This work
pEE2	pET16b *parC*-His	Amp, Cam	This work
pET16b	*bla, lac*I	Amp	Novagen
pGEM-T Easy Vector	*bla, lac*Z	Amp	Promega
pIF01	pFH2102 *par*C	Amp	Flåtten

Primers

Table A1.3 *Primers used in this thesis*

Primer	Nucleotide sequence	Producer
ParC F NcoI-His	5`- CGT TA CC ATG GGC CAT CAT CAT CAT CAT CAC ATG AGC GAT ATG GCA GAG CG - 3`	DNA Technology
ParC R BamHI	5`- CGT CTC TA GGA TCC TTA CTC TTC GCT ATC ACC GC TGC- 3`	DNA Technology
parC + 542	5`- GCA TAA CCT GCG TGA AGT GG - 3`	DNA Technology
parC + 1192	5`- GAA ACC GGC GCT GAT GTC G - 3`	DNA Technology

Antibiotics

Table A1.4 *Stock solutions of antibiotics used in this thesis*

Antibiotic	Stock solution	Producer
Ampicillin (Pentrexyl®)	100 mg/ml in dH_2O	Bristol-Myers Squibb
Chloramfenicol	30 mg/ml in CH_3COOH	Sigma

Enzymes and proteins

Table A1.5 *Restriction enzymes used in this thesis*

Enzym	Restriction site (5´ → 3´)	Buffer	Producer
Bam HI	G/GATCC	NEBuffer for BamHI	New England Biolabs Inc.
NcoI	C/CATGG	NEBuffer 4	New England Biolabs Inc.

Table A1.6 *Other enzymes and proteins used in this thesis*

Enzym/protein	Buffer	Producer
AmpliTaq polymerase	10 X PCR Buffer	Applied Biosystems
Anti-Rabbit IgG (whole molecule) Cy3 Conjugate		Sigma

Bovine serum albumin (BSA)		New England Biolabs Inc.
Lysozyme from Chicken Egg White		Sigma|
Primary rabbit anti-ParC antibody		Department of Cell Biology
rLysozyme solution 30 KU/µl		Novagen
Secondary anti-rabbit IgG		Sigma
T4 DNA ligase (400U/µl)	10 x Buffer for T4 DNA ligase	New England Biolabs Inc.

Markers

Table A1.7 *Markers used in this thesis*

Marker	Producer
2-Log DNA Ladder	New England Biolabs Inc.
Precision Plus Protein™, Dual Color Standards	BioRad

Chemicals

Table A1.8 *Chemicals used in this thesis*

Chemical	Producer
Acetic acid	Merck
Acrylamide/Bis Solution 30 %	Bio-Rad
Agarose, Type 1	Sigma
APS (ammonium persulfate)	Sigma
β-mercaptoethanol	Stratagene
Bromophenol blue	Oxoid
$CaCl_2$	Sigma
Calf thymus DNA Ultrapure	Sigma
CH_3COOH	Arcus
Chloroform	Aldrich-Chemie
Coomassie® brilliant blue R-250	Bio-Rad
Cyanogen bromide-activated-Sepharose®, 6 MB (Macrobead	Amersham Pharmacia Biotech
Decon 90	Decon Laboratories
Deconex	Decon Laboratories

dNTP mix 100mM, (dATP, dCTP, dGTP, dTTP)		Applied Biosystems
DTT (Dithiotreitol)		Sigma
EDTA (ethylene-diamine-tetra-acetate)		Sigma
Ethidium bromide 1 % in water		Sigma
Glucose		Sigma
Glycerol		Sigma
Glycin		Sigma
HCl		Merck
Hoechst 33258		Sigma
IPTG, (Isopropyl-β-D-1-thiogalactopyranoside)		Sigma
Isopropanol		Merck
KCl		Merck
KH_2PO_4		Sigma
Methanol pro analysi		Merck
Natrium Acetate		Sigma
NaCl		Sigma
$NaHCO_3$		Sigma
Na_2HPO_4		Merck
NaOH		Merck
N_2(l)		Aga
pH calibration solutions	pH 4.005	Radiometer Analytical
	pH 7.000	Radiometer Analytical
	pH 10.012	Radiometer Analytical
Phenol > 5 %		Sigma
Poly-$_L$-Lysine Solution		Sigma
Protein A Sepharose® CL-4B		Pharmacia Biotech
SDS (sodium dodecyl sulfate)		Serva
Temed N,N,N´,N´-Tetra-methyl-ethylenediamine		Bio-Rad
Tris-hydroxymethyl-aminomethan		Sigma
Tween® 20		Merck-Schucardt
X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)		Sigma

Commercial products

Table A1.9 *Commercial products used in this thesis*

Product	Producer
BCA Protein Assay Reagent Kit	Pierce
BugBuster® Ni-NTA His-Bind® Purification Kit	Novagen
QIAquick® Gel Extraction Kit	QIAGEN
QIAquick® PCR Purification Kit	QIAGEN
pGEM-T Easy Vector System II	Promega
Pure Yield™ Plasmid Midiprep System	Promega
Wizard®*Plus* SV Gel and PCR Clean-Up System	Promega
Wizard®*Plus* SV Miniprep	Promega

Equipments

Table A1.10 *Equipments used in this thesis*

Equipment	Model	Producer
Balances	Precision Standard	Ohaus Corp.
	CP 64	Sartorius
Bio-imazing system	Chemigenius	Synagene
CCD camera	MicroMax	Princeton Instruments Inc.
Centrifugal filter devices	Centricon®	Amicon bioseparations
Centrifuges	Biofuge 13	Heraeus Instruments
	Eppendorf centrifuge 5417R	Eppendorf
	Galaxy minicentrifuge	VWR International
	KUBOTA 5900	Kubota Corp.
	KUBOTA 5910	Kubota Corp.
	Sorvall RC 5C *Plus Rotor*	Du Pont Institute
Cover slip 18 x 18 mm		Assistant
Cover slip 24 x 24 mm		Assistent
Dialysis membrane cassette	Slide-A-Lyzer®	Pierce
DNA quantification apparatus	Hoefer® DyNA Quant 200	Amersham Pharmacia Biotech
Electric power supply	PowerPac 300	BioRad
	PowerPac 3000	BioRad
Electrophoresis chamber	Modell B1	OwI Separation Systems, Inc.

Electroporation apparatus	Gene Pulser® II	
	Pulse Controller Plus	BioRad
	Capitance extender	BioRad
Electroporation cuvette	Gene Pulser® cuvette 0.2 cm	BioRad
End-over-end shaker	Labquake Shaker	Labindustries, Inc.
Excitation filter for Cy3	BP546/12	Zeiss
Excitation filter for Hoechst 33258	BP365/12	Zeiss
Filter paper 3 (immunoblotting)	Whatman 3 MM paper	Whatman
Fluorescence/phase contrast	Axioplan 2	Zeiss
Fluorescenct scanning instrument	ChemiGenius2	Syngene
FPLC apparatus	Gradient Programmer GP-250	Pharmacia Fine Chemicals
	Pump P-500	Pharmacia Fine Chemicals
	Fraction Collector Frac-100	Pharmacia Fine Chemicals
	Single path monitor UV-1	Pharmacia Fine Chemicals
	Recorder 220 V	Pharmacia Fine Chemicals
Gelfiltration column	Superdex™ 200 10/300 GL	GE Healthcare
Heatblock	QBT1	Grant Instruments
Heatcloset		Termaks
Laboratory film	Parafilm “M”	American National Can™
Lens-cleaning tissue		Assistant
Lid for Microplate, sterile		Greiner Bio-One
Magnetstirrer	RCT basic	IKA® Werke
Microscope slides	Cut edges/frosted end	Menzel-Gläser
Microwave oven		Sharp
Microwell plate	PS Microplate 96 well	Greiner Bio-One
Microwell plate reader	Original Multiscan EX	Labsystems
Minishaker	MS2 *Minishaker*	IKA® Works, Inc.
PCR machine	PTC-100 ™ Programmable Thermal Controller	MJ Research Inc.
	Mastercycler gradient	Eppendorf
pH- meter	inoLab pH-meter	Semat
Pipettes	Pipetboy comfort	IBS Integra biosciences
	Eppendorf	Eppendorf
Plate scanner	Multiskan EX	Labsystem
Platform incubator	Thermomixer comfort	Eppendorf

Platform shaker	Platform shaker str6	Stuart Scientific
Polaroid camera	GelCam	Polaroid
	Electrophoresis hood 0.4 X	Polaroid
	Electrophoresis hood 0.8 X	Polaroid
Polaroid film	667 3 $^{1}/_{4}$ X 4 $^{1}/_{4}$ Film-pack	Polaroid
PVDF membrane	Immobilon™-P	Millipore
Rotors for Sorvall	GS-3	Du Pont Institute
Semi-dry blotter	Trans-Blot® SD Semi-dry transfer cell	BioRad
Shaking incubator	Orbital Incubator	Gallenkamp
Sterile 0.22 µm filter	Express™ PLUS	Millipore
Spectrophotometer	UV- 160 A	Shimadzu
UV- illuminator	TFX-20.M	Vilber Lourmat
Vacuum manifold		Promega
Water bath	Maxi-shake	Heto
	Comfort Heto Master Jet	Heto
	Shaking water bath SBS40	Stuart

Software

Table A1.11 *Software used in this thesis*

Software	Producer
Adobe Acrobat	Adobe Systems Inc.
Adobe Photoshop CS2	Adobe Systems Inc.
AxioVision version 3.0.6	Zeiss
AxioVision Image Viewer	Zeiss
BioEdit Sequence Alignment Editor	
GeneSnap	Synoptics Ltd.
Gene Tools	Synoptics Ltd.
Microsoft Office Excel 2003	Microsoft
Microsoft Office Powerpoint 2003	Microsoft
Microsoft Office Word 2003	Microsoft
Reference Manager Version 11	ISI ResearchSoft
Vector NTI Advance 10	Invitrogen

Growth media

LB (Luria-Bertani)

Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
dH20	to 1000 ml

Low salt LB contain 5 g NaCl

Adjust pH to 7.0 with NaOH and then autoclave.

LB Agar

Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
Agar	20 g
dH20	to 1000 ml

Adjust pH to 7.0 with NaOH and then autoclave.

Pour into Petri dishes (~25 ml/100-mm plate)

AB Minimal medium

5xA

$(NH_4)_2SO_4$	2 g
Na_2HPO_4	6 g
KH_2PO_4	3 g
NaCl	3 g
dH_2O	to 200 ml

B

0.1 M $CaCl_2$	1 ml
1.0 M $MgCl_2$	1 ml
0.003 M $FeCl_3$	1 ml
Autoclaved H_2O	to 800 ml

5xA and B were mixed. This gave AB.

1 ml 1 mg/ml thiamine was added to AB. This made ABB_1.

ABB_1 was supplemented with:

0.2 % glucose

0.5 % CAA

100 µg/ml uridine.

Buffers and solutions

TE-buffer (~pH 8)

Tris-HCl	10 mM
EDTA	1 mM

Agarose gel electrophoresis

5 x TBE buffer

Tris base	54 g
Boric acid	27,5 g
0.5 M EDTA	20 ml
dH2O	to 1000 ml

The agarose gels were run in 0.5x TBE buffer.

Agarose gel loading buffer

87 % glycerol	500 µl
dH2O	500 µl
Bromophenolblue	0,03 %

Coomassie staining solutions

Coomassie

Coomassie Brilliant Blue R-250	2.5 g
Methanol	455 ml
dH_2O	455 ml
Acetic acid	90 ml

40 % methanol/ 20 % acetic acid

Methanol	400 ml
Acetic acid	200 ml
dH_2O	400 ml

Proteinmåling

BSA Std 2 mg/ml

Coomassie Plus Protein Assay Reagent

DNA quantification buffer

10 x TNE buffer

Tris base	100 mM
EDTA	10 mM
NaCl	2 M

10 x TNE buffer was diluted to 1 x TNE buffer and added 1:10000 1 mg/ml Hoechst 33258

Gel filtration buffer

GF buffer 1

Tris-HCl pH 7.66 (v/5°C)	20 mM
EDTA	0.2 mM

GF buffer 2

Tris-HCl pH 7.66 (v/5°C)	20 mM
EDTA	0.2 mM
NaCl	0.5 M

Immunostaining

PBS buffer

5 M NaCl	7 ml
4 M KCl	125 µl
0.1 M Na_2HPO_4	20 ml
1 M KH_2PO_4	375 µl
dH_2O	to 250 ml

PBS-T contained 0.05 % Tween

Lysozyme solution

30 mg/ml lysozyme		133 µl
1 M Tris-HCl		50 µl
50 µl % glucose		36 µl
0.5 M EDTA		4 µl
dH_2O	to	2000 µl

Mounting medium antifade

1 M PBS		20 µl
87 % glycerol		460 µl
1 mg/ml Hoechst 33258		5 µl
dH_2O	to	1000 µl

Protein purification buffers under denaturing conditions

1 M NaH_2PO_4/ Na_2HPO_4 pH 8

1 M NaH_2PO_4	6.8 ml
1 M Na_2HPO_4	93.2 ml

1 M NaH_2PO_4/ Na_2HPO_4 pH 6.3

1 M NaH_2PO_4	82.2 ml
1 M Na_2HPO_4	17.8 ml

1 M NaH_2PO_4/ Na_2HPO_4 pH 5.9

1 M NaH_2PO_4	92.1 ml
1 M Na_2HPO_4	7.9 ml

1 M NaH_2PO_4/ Na_2HPO_4 pH 4.5

1 M NaH_2PO_4	98.0 ml
1 M Na_2HPO_4	2.0 ml

Denaturing lysis/bind buffer - Buffer B

Urea	48.05 g
1 M NaH_2PO_4/ Na_2HPO_4 pH 8	10 ml
1 M Tris- HCl pH 8	1 ml
dH_2O	to 100 ml

Denaturing wash buffer - Buffer C

Urea	48.05 g
1 M NaH_2PO_4/ Na_2HPO_4 pH 6.3	10 ml
1 M Tris- HCl pH 6.3	1 ml
dH_2O	to 100 ml

Denaturing elution buffer - Buffer D

Urea	48.05 g
1 M NaH_2PO_4/ Na_2HPO_4 pH 5.8	10 ml
1 M Tris- HCl pH 5.8	1 ml
dH_2O	to 100 ml

Denaturing elution buffer - Buffer E

Urea	48.05 g
1 M NaH_2PO_4/ Na_2HPO_4 pH 4.5	10 ml
1 M Tris- HCl pH 4.5	1 ml
dH_2O	to 100 ml

Due to the dissociation of urea, the pH of Buffers B, C, D and E should be adjusted immediately prior to use.

Dialyse Buffer with 4 M Urea

Urea	240.24 g
1 M Tris-HCl pH 7.5	50 ml
0.5 M EDTA	2 ml
1 M DTT	5 ml
5 M NaCl	40 ml
99 % Glycerol	300 ml
dH_2O	to 1000 ml

Dialyse Buffer with 2 M Urea

Urea	120.12 g
1 M Tris-HCl pH 7.5	50 ml
0.5 M EDTA	2 ml
1 M DTT	5 ml
5 M NaCl	40 ml
99 % Glycerol	300 ml
dH_2O	to 1000 ml

Dialyse Buffer without Urea

Similar to native conditions

Protein purification buffers under native conditions

1 M NaH_2PO_4/ Na_2HPO_4 pH 8

1 M NaH_2PO_4	6.8 ml
1 M Na_2HPO_4	93.2 ml

Bind Buffer

NaCl	876 mg
1 M NaH_2PO_4/ Na_2HPO_4 pH 8	2.5 ml
Imidazol	34.4 mg
dH_2O	to 50 ml

Wash Buffer A

NaCl	876 mg
1 M NaH_2PO_4/ Na_2HPO_4 pH 8	2.5 ml
Imidazol	68.8 mg
dH_2O	to 50 ml

Wash Buffer B

NaCl	876 mg
1 M NaH_2PO_4/ Na_2HPO_4 pH 8	2.5 ml
Imidazol	137.6 mg
dH_2O	to 50 ml

Elution Buffer

NaCl	876 mg
1 M NaH_2PO_4/ Na_2HPO_4 pH 8	2.5 ml
Imidazol	860 mg
dH_2O	to 50 ml

Dialyse Buffer

1 M Tris-HCl pH 7.5	50 ml
0.5 M EDTA	2 ml
1 M DTT	5 ml
5 M NaCl	40 ml
99 % Glycerol	300 ml
dH_2O	to 1000 ml

Coupling buffer

0.1 M $NaHCO_3$

0.5 M NaCl

pH 8.3-8.5

SDS-polyacrylamide gel electrophoresis

2 x SDS gel sample buffer

5 % Bromophenol blue	0.4 ml
1 M Tris-HCl pH 6.5	1.0 ml
1 M DTT	2.0 ml
Glycerol 99 %	2.02 ml
10 % SDS	4.0 ml
dH_2O	0.58 ml

1.5 M Separation gel buffer

Tris base	182 g
HCl	to pH ~ 8.8
dH2O	to 1000 ml

0.5 M Stacking gel buffer

Tris base	61 g
HCl	to pH ~ 6.8
dH2O	to 1000 ml

Separation gel solution (10 %)

dH_2O	2.0 ml
Separation gel buffer	1.25 ml
Acrylamide/Bis Solution (30 %)	1.7 ml
10 % SDS	50 µl
10 % APS	25 µl
TEMED	2.5 µl

Stacking gel solution (4 %)

dH_2O	875 µl
Stacking gel buffer	400 µl
Acrylamide/Bis Solution (30 %)	200 µl
10 % SDS	16 µl
10 % APS	8 µl
TEMED	1.6 µl

Running buffer 1 %

10 % SDS	20 ml
10 X Running buffer	200 ml
dH_2O	1780 ml

Immunoblot analysis buffers

Anode buffer I

Tris base		36.3 g
Methanol		200 ml
dH_2O	to	1000 ml

Anode buffer II

Tris base		3.03 g
Methanol		200 ml
dH_2O	to	1000 ml

Cathode buffer

6-amino-n-hexanoic acid	5.2 g
Methanol	200 ml
dH_2O	to 1000 ml

5xTBS-buffer

1 M Tris- HCl (pH7.5)	50 ml
NaCl	146 g
dH_2O	to 1000 ml

TBS-T contained 0.05 % Tween.

Small scale DNA plasmid purification solutions

Alkaline lysis solution I

Glucose	50 mM
Tris- HCl (pH 8.0)	25 mM
EDTA (pH 8.0)	10 mM

Alkaline lysis solution II

NaOH	0.2 M
SDS	1 %

Alkaline solution II should be freshly prepared.

Alkaline lysis solution III

Potassium acetate 5M	60 ml
Acetic acid	11.5 ml
dH_2O	to 100 ml

APPENDIX 2

Protocols for standard DNA techniques

Small scale DNA plasmid purification

Small scale DNA plasmid purification was performed using Wizard® *Plus* SV Minipreps DNA Purification Systems (Promega)
(Product instructions for Wizard® SV Plus Minipreps DNA Purification Systems, Promega)

1. 4.5 ml of an overnight (12-16 hours) culture grown in LB medium, containing 50 µg/ml ampicillin and 30 µg/ml chloramphenicol, was centrifuged at maximum speed (13 000 rpm) for 1 minute. The supernatant was removed and the tube was inverted on a paper towel to remove excess media.
2. 250 µl Cell Resuspension Solution was added and the cell pellet resuspended thoroughly by pipetting and vortexing.
3. 250 µl Cell Lysis Solution was added and the tube was inverted 4 times to mix the solution. The tube was incubated 1-5 minutes until the solution was clear.
4. 10 µl Alkaline Protease Solution was added, mixed by inverting the tube 4 times and incubated for 5 minutes.
5. 350 µl Neutralization Solution was added, the tube inverted 4 times and the solution centrifuged at maximum speed for 10 minutes.
6. The Spin Column was inserted into a Collection Tube. The cleared lysate was transferred carefully to the prepared Spin Column by decanting.
7. The supernatant was centrifuged at maximum speed for 1 minute. The Spin Column was removed from the tube, the flowthrough was discarded and the Spin Column was reinserted into the Collection Tube.

8. 750 µl Wash Solution was added to the Spin Column. The solution was centrifuged at maximum speed for 1 minute, the Spin Column removed and the flowthrough discarded. Spin Column was reinserted into the Collection Tube.
9. The wash procedure was repeated using 250 µl Wash Solution. The solution was centrifuged at maximum speed for 2 minutes.
10. The Spin Column was carefully transferred to a new sterile centrifuge tube.
11. Plasmid DNA was eluted by adding 100 µl of Nuclease-Free Water to the Spin Column. The column was centrifuged at maximum speed for 1 minute.
12. After the plasmid DNA was eluted the Spin Column was discarded.
13. The DNA concentration was measured as described in paragraph 2.2.3
14. The purified plasmid DNA was stored at -20 °C.

APPENDIX 3

Large scale DNA plasmid purification

Large scale DNA plasmid purification was performed using PureYield™ Plasmid Midiprep Systems (Promega)

(*Product instructins for Pure Yield™ Plasmid Midiprep Systems, Promega)*

1. 50 ml of an overnight culture grown in LB medium, containing 50 µg/ml ampicillin and 30 µg/ml chloramphenicol, was centrifuged at 6 000 rpm for 10 minutes. The supernatant was removed and the tube was inverted on a paper towel to remove excess media.
2. The cells were resuspended in 3 ml Cell Resuspension Solution.
3. 3 ml Cell Lysis Solution was added, the tube gently inverted 3-5 times and then incubated at room temperature for 3 minutes.
4. 5 ml Neutralization Solution was added to the lysed cells and the tube was inverted 5 times. The tube was incubated 2-3 minutes in an upright position to allow a white flocculent precipitate to form.
5. The lysate was poured into the PureYield™ Clearing Column and incubated for 2 minutes to allow the cellular debris to rise to the top.
6. The lysate was centrifuged in a swinging bucket rotor centrifuge at 1 500 x g for 5 minutes. The tube should not be capped during centrifugation.
7. A PureYield™ Binding Column was placed into a vacuum manifold
8. The lysate was poured into the binding column and applied maximum vacuum. The vacuum was continued until all the liquid had passed through the binding column.
9. The vacuum was slowly released from the binding column.
10. 5.0 ml of Endotoxin Removal Wash was added to the binding column, and the solution was pulled through the binding column by vacuum.

11. 20 ml of Column Wash Solution was added to the binding column and the solution was pulled through the binding column by vacuum.
12. The membrane was dried by applying a vacuum for 30 seconds.
13. The binding column was removed from the vacuum manifold and tapped on a paper towel to remove excess ethanol. Excess ethanol was wiped from the outside of the tube and the binding column was placed into a new 50 ml disposable plastic tube.
14. 600 µl of Nuclease-Free water was added to the DNA binding column. The binding column was centrifuged in a swinging rotor centrifuge at 1 500 x g for 5 minutes and the filtrate collected.
15. The purified DNA solution was stored at -20°C
16 The DNA concentration was measured as described in paragraph 2.2.3

APPENDIX 4

Purification of DNA from gel bands

Purification of DNA from gel bands was preformed using QIAquick® Gel Extraction Kit (QIAGEN) and Wizard® SV Gel and PCR Clean-Up System (Promega) (*Product instructions for QIAquick® Gel Extraction Kit (QIAGEN) and Wizard® SV Gel and PCR Clean-Up Systems (Promega))*

QIAquick® Gel Extraction Kit

1. An empty 1.5 ml eppendorf tube was weighed and the weight was recorded.
2. The DNA fragment was excised from the agarose gel with a clean scalpel. Extra agarose was minimized around the DNA band by cutting and the slice was transferred to the pre-weighed eppendorf tube.
3. The gel sliced was weighed in the tube and the weight of the empty tube was subtracted to determine the weight of the slice. The maximum amount of gel slice per QIAquick® column was 400 mg.
4. 3 volumes of Buffer QG were added to 1 volume of gel slice.
5. The eppendorf tube was closed and incubated at 50°C for 10 minutes until the gel was completely dissolved.
6. A QIAquick® spin column was placed in a provided 2 ml collection tube.
7. The sample was transferred to the QIAquick® column, and centrifuged at 13 000 rpm for 1 minute at room temperature.
8. The flow-through was discarded and the QIAquick® column was placed back in the same collection tube.
9. To remove all traces of agarose 0.5 ml Buffer QG was added to QIAquick® column and centrifuged for 1 minute at room temperature.

10. 0.75 ml of Buffer PE was added to QIAquick® column to wash. The sample was incubated 2 minutes before it was centrifuged at 13 000 rpm for 1 minute at room temperature.
11. The flow-through was discarded and the QIAquick® column centrifuged for an additional 1 minute at 13 000 rpm.
12. The collection tube was discarded and the QIAquick® column was placed into a clean 1.5 ml eppendorf tube.
13. To elute the DNA, 30 µl of autoclaved H_2O was applied to the center of the QIAquick® membrane.
14. The sample was incubated at room temperature for 1 minute.
15. The sample was centrifuged at 13 000 rpm at room temperature for 1 minute.
16. The sample was stored at -20°C.

Wizard® SV Gel and PCR Clean-Up System

1. Following electrophoresis, The DNA band was excised from the agarose gel and placed in a pre-weighed eppendorf tube.
2. The tube with the gel sliced was weighed, the weight of the tube subtracted and 10 µl Membrane Binding Solution was added per 10 mg of gel slice.
3. The tube was vortexed and incubated at 50°C until the gel slice was completely dissolved.
   For PCR reactions an equal volume of Membrane Binding Solution was added.
4. SV Minicolumn was inserted into Collection Tube.
5. The dissolved gel slice or the PCR product was transferred to the Minicolumn assembly, and incubated for 1 minute at room temperature.
6. The sample was centrifuged at 13 000 rpm for 1 minute at room temperature. The flowthrough was discarded and the Minicolumn reinserted into Collection Tube.
7. 700 µl Membrane Wash Solution was added and centrifuged at 13 000 rpm for 1 minute at room temperature. The flow-through was discarded and the Minicolumn reinserted into Collection Tube.

8. Step 7 was repeated with 500 µl Membrane Wash Solution and centrifuged at 13 000 rpm for 5 minutes at room temperature.
9. The Collection tube was emptied and the column assembly was recentrifuged for 2 minutes to allow evaporation of residual ethanol.
10. The Minicolumn was transferred to a clean 1.5 ml eppendorf tube.
11. 50 µl of Nuclease-Free water was added to the Minicolumn, incubated at room temperature for 1 minute and centrifuged at 13 000 rpm for 1 minute.
12. The Minicolumn was discarded the DNA stored at -20°C.

APPENDIX 5

Agarose gel electrophoresis

(Sambrook and Russell, 2001)

1. The specified amount of powdered agarose was weighed and solved in 50 or 60 ml 0.5 x TBE-buffer. The solution was heated in a microwave until the agarose dissolved.
2. The warm gel solution was poured into the mold, and the appropriate comb positioned for forming the sample slots in the gel.
3. The gel was allowed to set completely (30-45 minutes at room temperature), the comb was removed and the gel mounted in the electrophoresis tank filled with enough 0.5 x TBE buffer to cover the gel.
4. The DNA samples and the DNA marker were mixed with the desired gel-loading buffer, and slowly loaded into the slots.
5. The gels were run at 90-100 V until the samples had migrated a sufficient distance through the gel.
6. The gel was stained by immersing it in H_2O containing EtBr, for 30-45 minutes in room temperature, in a dark room.
7. The DNA was visualized with UV light and photographed with a Polaroid camera or with GeneSnap

APPENDIX 6

The polymerase chain reaction (PCR)

1. 4 µl of an overnight culture (pIF01) grown in LB was added to 96 µl PCR mix containing 1 x PCR buffer, 200 µM dNTP, 2 pM primer ParC F NcoI, and primer ParC R BamHI and 5 U Ampli Taq Gold.
2. The PCR reaction was carried out after the following program:

Initial Denaturation (Hot start)	94ºC	2 minutes
Denaturation	94ºC	30 seconds ⎫
Annealing	60ºC	30 seconds ⎬ 30 cycles
Synthesis	68ºC	2 minutes ⎭
Extension	68ºC	5 minutes

3. The PCR product was identified on a 1 % agarose gel as described in appendix 5, and the DNA was purified as described in appendix 4.

APPENDIX 7

Digestion of DNA by restriction nucleases

1. 1-10 µg of purified plasmid DNA or PCR product was mixed with the appropriate 10 x reaction buffer diluted to 1 x reaction buffer with dH_2O. BSA was added to a final concentration of 100 µg/ml.
2. 5-20 U/µg DNA of restriction nucleases was added to 50-100 µl reaction mixture.
3. The sample was thoroughly mixed by pipetting up and down and shortly spinned to collect the sample in the bottom of the tube.
4. The sample was incubated at 37ºC for 2-3 hours.
5. The restriction nuclease, NcoI was inactivated at 65ºC for 20 minutes. BamHI could not be inactivated.
6. The digested fragments were run on a 0.8 % agarose gel as described in appendix 5 and purified as described in appendix 4.

Ligation

1. The amplified DNA insert and the plasmid vector were digested with restriction nucleases as described in paragraph 2.3.2 and mixed in a molar ratio of 2:1 in 10 x T4 ligase buffer diluted (with dH_2O if necessary) to 1 x ligase buffer to a total reaction volume of 20 µl.
2. 1 µl T4 DNA ligase (400 U/ µl was added to the reaction mixture.
3. The reaction mixture was incubated at 16ºC over night.

APPENDIX 8

Preparation of electrocompetent cells

1. 3 x 1 ml ON culture of DH5α cells was made
2. ON culture was diluted 1:100 in 320 ml low-salt LB in a 2.5 l bottle. The cells were grown until OD_{600} reached 0.8 with vigorous shaking at 37ºC.
3. The cells were transferred to 50 ml centrifuge bottles and placed on ice for 15-30 min. The bottles were centrifuged at 4 000 rpm for 15 minutes at 4°C
4. The media was removed and the cells resuspended in 400 ml cold dH_2O. The bottles were centrifuged at 4 000 rpm for 15 minutes at 4°C.
5. The media was removed and the cells resuspended in 200 ml cold dH_2O. The bottles were centrifuged at 4 000 rpm for 15 minutes at 4°C.
6. The media was removed and the cells resuspended in 8 ml 10 % cold glycerol. The bottles were centrifuged at 4 000 rpm for 15 minutes at 4°C.
7. The media was removed and the cells resuspended in 2.4 ml 10 % cold glycerol. (Used twice as much glycerol due to “tett kultur”)
8. The cell mixture was divided in aliquots of 100 µl in eppendorf tubes and frozen at -80°C.

APPENDIX 9

Electroporation

1. The cells were thawed carefully and kept on ice.
2. 1-2 µl DNA was added and mixed well. The mixture was kept on ice for 1 minute.
3. Gene Pulser™ Apparatus settings were adjusted to:

Capacitance:	25 µF
Voltage:	2.5 kV
Pulse Controller:	400 Ω

4. The mixture of electrocompetent cells and DNA was transferred to a cold 0.2 cm Gene Pulser Cuvette and put in the cuvette holder. The holder was pushed into the chamber between the electrodes.
5. The pulse was started by pushing both the red knob on the Gene Pulse Apparatus simultaneously until a beep sound was heard. This gave a pulse of 4-5 msec. and field strength of 12.5 kV/cm.
6. The cuvette was removed from the chamber and 1 ml LB medium was added to the cuvette immediately. The cells were resuspended with a pasteur pipette, and transferred to a 15 ml sterile plastic tube. Then the cells were incubated at 37 °C for 1 hour with agitating.
7. 10 µl and 100 µl were spread on LB agar plates containing 50 µg/ ml ampicillin.
8. The plates were incubated at 37°C over night for colony formation.

APPENDIX 10

Preparation of chemically competent cells using $CaCl_2$

1. 2 x 1 ml LB medium was inoculated with DH5α and grown ON at 37°C with agitating.
2. 0.5 ml ON culture was inoculated with 25 ml fresh LB.
3. When OD_{600} reached 0.3 the tube was put on ice for 10 minutes.
4. The tube was centrifuges at 5 000 rpm for 10 minutes at 4°C.
5. The supernatant was discarded and the cells resuspended in 1 ml ice-cold $CaCl_2$/glycerol [0.1M/15%]
6. The cells were placed on ice for 30 minutes or more (the longer the better)
7. The cell mixture was divided in aliquots of 200 µl in eppendorf tubes.
8. The cells were frozen at -80°C.

Transformation of chemically competent cells ($CaCl_2$)

1. The cells were thawed on ice.
2. 4 µl ligation mixture was added to 200 µl competent cells.
3. The mixture was placed on ice for 30 minutes.
4. The tube was heat-pulsed at 42°C for 90 sec, and then placed on ice for 2 minutes.
5. 0.8 ml LB was added and incubated at 37°C for 45-60 minutes. The tube was vigorously shaken.
6. The mixture was spread on LB agar plate containing 50 µg/ml ampicillin.
7. The plate was incubated at 37°C overnight.
8. Positive transformants were selected as described below in appendix 12 and kept in glycerol stocks at -80°C for further use.

APPENDIX 11

Transformation of "ultra-competent" cells

1. A 14 ml Falcon tube was pre-chilled on ice. LB medium was preheated to 42°C.
2. The cells were thawed on ice, gently mixed and 100 µl of cells were put in the tube.
3. 4 µl β-ME mix (β-mercaptoethanol) was added to the cells
4. The tube was swirled gently and the cells were incubated on ice for 10 minutes, swirling gently every 2 minutes.
5. 2 µl ligation mixture was added to the cells, and the tube was swirled and incubated on ice for 30 minutes.
6. The tube was heat-pulsed in a 42°C water bath for 30 seconds.
7. The tube was incubated on ice for 2 minutes.
8. 0.9 ml of preheated LB medium was added and the tube incubated at 37°C for 1 hour with shaking at 225-250 rpm.
9. The transformation mixture was plated on LB agar plate containing ampicillin 50 µg/ml and chloramphenicol 30 µg/ml.
10. The plate was incubated at 37°C overnight.
11. Positive transformants were selected as described in below in appendix 12 and kept in glycerol stocks at -80°C for further use.

APPENDIX 12

Selection of transformants

1. 4-5 colonies were picked from the LB agar plates and each colony was inoculated in 6 ml LB medium containing appropriate antibiotics, and incubated with vigorous shaking at 37°C over night.
2. Small scale DNA plasmid purification was performed on possibly positive transformants as described in appendix 2.
3. The samples were digested with restriction enzymes as described in appendix 7, and identified by agarose gel eletrophoresis as described in appendix 5.
4. Positive transformant were selected and kept in glycerol stocks at -80°C for further use.

APPENDIX 13

Subcloning

1. The pGEM®T-Easy Vector tube was briefly centrifuged to collect the content at the bottom of the tube.
2. A ligation reaction was set up:

2 x Rapid Ligation Buffer, T4 DNA Ligase	5 µl
pGEM®T Easy Vector (50 ng)	1 µl
PCR product (120 ng)	1 µl
T4 DNA Ligase (3 Weiss units/µl)	1 µl
Deionized water to a final volume of	10 µl

APPENDIX 14

Transformation of protein expression cells

(*Product instruction for BL21-Gold(DE3)pLysS Competent Cells (Stratagene))*

1. The cells were thawed on ice.
2. A 14 ml Falcon tube was prechilled on ice and 100µl of the competent cells were gently mixed and added to the tube.
3. 50 ng of DNA was added to the transformation reaction and gently swirled.
4. The reaction was incubated on ice for 30 minutes.
5. LB medium was preheated in a 42ºC water bath.
6. The reaction was heat-pulsed in a 42ºC water bath for 20 seconds exactly.
7. The reaction was incubated on ice for 2 minutes.
8. 0.9 ml of preheated LB medium was added and the reaction was incubated at 37ºC for 1 hour with shaking at 225-250 rpm.
9. The reaction was plated onto LB plates with 50 µg/ml ampicillin and 30µg/ml chloramphenicol.
10. The plates were incubated at 37ºC overnight.

APPENDIX 15

Induction of target protein using IPTG

(*Product instruction for BL21-Gold(DE3)pLysS Competent Cells (Stratagene))*

1. 1 ml LB medium containing 50 µg/ml ampicillin and 30 µg/ml chloramphenicol was inoculated with single colonies from the transformation.
2. Next morning, 50 µl of each culture was inoculated into fresh LB medium containing no antibiotics. These cultures were incubated with shaking at 220-250 rpm at 37ºC for 2 hours.
3. 100 µl of each of the cultures were pipetted into clean micro centrifuge tubes and placed on ice until needed for gel analysis. These served as the non-induced control samples.
4. To the rest of the culture in each tube IPTG was added to a final concentration of 0.6 mM or 1 mM. The cultures were incubated with shaking at 220-250 rpm at 37ºC for 2 hours.
5. The cultures were centrifuged at 16 000 rpm for one minute. The supernatant was removed and the cell pellet frozen at -20ºC for 15-20 minutes.
6. Cell pellets were thawed and solved in 100 µl BugBuster. 2.5 U benzonase was added and the sample was incubated at room temperature for 10 minutes.
7. The samples were centrifuged at 16 000 rpm for 10 minutes and the supernatants were transferred to clean tubes.
8. The supernatants were compared on SDS- gel (appendix 17).

APPENDIX 16

Preparation of cell extract for SDS-PAGE

1. Cells from an overnight culture were grown to OD_{600} = 0.15 in LB or OD_{450} = 0.15 in AB minimal medium supplemented with 1μg/ml thiamine, 0.2 % glucose, 0.5 % casamino acids and 100 μg/ml uridine.
2. 10 ml of exponentially growing cells were centrifuged at 8 500 rpm for 5 minutes at 4°C.
3. The supernatant was removed and the pellet resuspended in 150 μl resuspension buffer.
4. The cells were lyzed at 98°C for 5 minutes.
5. The cells were stored at 4°C.

Measurement of total protein content

1. A set of diluted albumin BSA standards were prepared; 0 mg/ml, 0.5 mg/ml, 1.0 mg/ml, 1.5 mg/ml and 2.0 mg/ml, from 2.0 mg/ml BSA stock standard in the same diluent as the extract samples.
2. 10 μl of diluted BSA standards and 10 μl of cell extract samples were added to the appropriate microwell plate wells.
3. 200 μl of BCA Reagent A and BCA Reagent B (50:1) were added to each well and mixed well with a pipette tip.
4. The plate was covered and incubated at 37ºC for 30 minutes.
5. The plate was cooled to room temperature.
6. The absorbance was measured at 570 nm on a microwell plate reader.
7. A standard curve was made by plotting the absorbance for each BSA standard versus its concentration in μg/ml.
8. By using standard curve, the protein concentration of each unknown sample was determined.

APPENDIX 17

Preparation of SDS polyacrylamide gel and samples, and running the gel

1. Two glass plates were washed with 70 % ethanol and dH_2O and dried.
2. The glass plates were assembled together and the separation gel solution (10%) was poured on top, in between the glass plates.
3. 1 ml dH_2O was added to the top of the solution.
4. After polymerization was complete (~ 30 minutes), dH_2O was removed, and stacking gel solution (4%) was poured on top of the polymerized separation gel.
5. A 1.0 mm comb was immediately inserted into the stacking gel solution.
6. After polymerization was completed (~ 30 minutes), the comb was removed and the wells washed with dH_2O to removed any unpolymerized acrylamide.
7. The gel was mounted into the electrophoresis chamber.
8. Running buffer 1% was added to the top and bottom reservoirs.
9. A SDS-PAGE marker and samples were mixed with the appropriate amount of 2 x SDS gel-loading buffer, heated for 5 minutes at 98ºC and loaded into the wells.
10. The rest of the top reservoir of the electrophoresis chamber was completely filled with running buffer 1%.
11. The electrophoresis apparatus was attached to an electric supply and the gel was run at a constant voltage of 150 V for 1-1 ½ hours.

APPENDIX 18

Detection of proteins by immunoblot analysis

1. The PVDF membrane was cut in the correct size and the hydrophobic surface of the membrane was wet with methanol and then soaked in dH_2O for about 15 minutes.
2. 6 Whatman 3MM papers were cut in the correct size and saturated with the appropriate transfer buffer; 2 papers in Anode buffer I, 1 paper in Anode buffer II and 3 papers in Cathode buffer.
3. The saturated Whatman 3MM papers, the saturated PVDF membrane and the gel were placed in the semidry blotting apparatus in the in the following order relatively to each other; Anodebuffer I – Anodebuffer II – PVDF-membrane – gel – Cathode buffer. The membrane was placed underneath the gel facing the anode.
4. The semidry blotting apparatus was attached to an electric power supply and run at 15 V for 30-60 minutes.
5. The membrane was soaked in low-fat dried milk dissolved in TBS-T for at least 60 minutes with slow shaking in room temperature or overnight at 4ºC.

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6. The primary antibodies; anti-SeqA and ParC were diluted 10 000 fold and 200 fold, respectively in 15 ml low-fat dried milk dissolved in TBS-T.
7. The membrane was incubated with the primary antibody for 2 hours with slow shaking.
8. The membrane was washed 4 x 5 minutes with TBS-T.
9. The secondary antibody (anti-rabbit IgG) was diluted 10 000 fold in 15 ml low-fat dried milk dissolved in TBS-T.
10. The membrane was incubated with the secondary antibody for 2 hours with slow shaking.
11. The membrane was washed again as in step 8.
12. The washed blot with the protein-side up was placed on a clean glass plate.

13. 1 ml ECF substrate was added and the membrane was kept in a dark place for 5 minutes. Then the membrane was transferred to a Whatman 3MM paper to dry in the dark for another 30 minutes.
14. The membrane was scanned and viewed using GeneSnap software.

APPENDIX 19

Preparation of cell lysate for Ni^{2+}-column

1. 15 ml LB containing 50 μg/ml ampicillin and 30 μg/ml chloramphenicol was inoculated with the strain EE3. The culture was grown at 37°C with constant shaking for 15-16 hours.
2. The overnight culture were centrifuged at 6 000 rpm for 10 minutes at 4ºC (Sorvall, GS-3 rotor). The supernatant was removed and the pellets resuspended in 2 000 ml LB, 50 μg/ml ampicillin and 30 μg/ml chloramphenicol. The culture was grown to OD_{600} ~ 0.6 with constant shaking.
3. At OD_{600} ~ 0.6, 0.6 mM IPTG was added, and the culture allowed to grow for another 120 minutes.
4. The cells were harvested by centrifugation at 6 000 rpm for 10 minutes at 4°C (Sorvall, GS-3 rotor).
5. The supernatant was removed and the pellets frozen at -20°C.
6. The frozen cells were thawed on ice and completely resuspended in 40 ml room temperature BugBuster Protein Extraction Reagent by pipetting. 40 μl Benzonase Nuclease was added. The cell suspension was incubated on ice for 10 minutes.
7. The cell suspension was centrifuged at 3 000 rpm for 20 minutes at 4°C. The pellet was saved for inclusion body purification as described below. The supernatant was used as lysate in the protocol underneath.

Preparation of Ni^{2+}-column and purification under native conditions

(The procedure was carried out in a cold room)

1. 1 ml of 50 % Ni-NTA His•Bind® slurry was added to 4 ml of Bind Buffer and mixed gently in a Falcon tube.
2. The resin was allowed to settle by gravity and 4 ml of the supernatant was removed with a pipette.

3. The cleared lysate from the protocol above was added to the Ni-NTA His•Bind® slurry and mixed gently at 4°C for 60 minutes at an end-over-end shaker.
4. The lysate-Ni-NTA His•Bind® mixture was loaded into a column with the bottom outlet capped.
5. The bottom cap was removed and the column flow-through was collected.
6. The column was washed with 4 ml Wash Buffer A. The buffer was loaded on gently.
7. The column was washed with 4 ml Wash Buffer B.
8. The protein was eluted with 4x 0.5 ml Elution Buffer.
9. The flow-through, wash fractions and eluate were analyzed on SDS-PAGE as in paragraph 17.
10. The elution-fractions with most protein were dialyzed against 500 ml Dialyze Buffer overnight at 4°C with stirring. The protein was further dialyzed the next day for 6 hours in another 500 ml Dialyze Buffer.

Preparation of Ni^{2+}-column and purification under denaturing conditions

(The procedure was carried out in a cold room)

1. The cell pellet from the preparation of cell lysate (appendix 19) was resuspended in 4 ml BugBuster Protein Extraction Reagent and mixed by pipetting up and down to obtain an even suspension. Thorough resuspension of pellet is critical to obtain a high purity inclusion body preparation. The inclusion body pellet was completely dispersed during BugBuster wash steps in order to solubilize and remove contaminating proteins from the pellet.
2. rLysozyme Solution was added to a final concentration of 1 KU/ml. The mixture was vortexed and incubated at room temperature for 5 minutes.
3. 10 volumes of BugBuster reagent diluted 1:10 (= 40 ml) in deionized water was prepared.
4. 6 volumes (= 24 ml) diluted BugBuster reagent was added to the suspension and vortexed for 1 minute.

5. The inclusion bodies were harvested by centrifugation at 5 000 x g for 15 minutes at 4°C and the supernatant was removed with a pipette.
6. The inclusion bodies were resuspended in 12 ml diluted BugBuster, mixed by vortexing and centrifuged at 5 000 x g for 15 minutes at 4°C. This wash step was repeated twice. The inclusion bodies were resuspended once more but centrifuged at 16 000 x g for 15 minutes at 4°C, and the supernatant removed.
7. The final pellet of purified inclusion bodies was resuspended in denaturing lysis/bind buffer; Buffer B
8. 1 ml of 50 % Ni-NTA His•Bind® slurry was added 4 ml of denaturing lysis/bind Buffer B and mixed gently in a Falcon tube.
9. The resin was allowed to settle by gravity and 4 ml of the supernatant was removed with a pipette.
10. 4 ml of lysate from step 7 was added to the Ni-NTA His•Bind® slurry and mixed gently at 4°C for 60 minutes at an end-over-end shaker.
11. The lysate-Ni-NTA His•Bind® mixture was loaded into a column with the bottom outlet capped.
12. The bottom cap was removed and the column flow-through was collected.
13. The column was washed with 4 ml denaturing wash buffer; Buffer C twice. The buffer was loaded on gently.
14. The protein was eluted with 2x0.5 ml denaturing elution buffer; Buffer D.
15. The protein was eluted with 2x0.5 ml denaturing elution buffer; Buffer E.
16. The flow-through, wash fractions and eluate were analyzed on SDS-PAGE as in paragraph 17.
17. The elution-fractions with most protein were dialyzed against 500 ml Dialyze Buffer with 4 M Urea overnight at 4°C with stirring. The protein was further dialyzed the next day for 4 hours in 500 ml Dialyze Buffer with 2 M Urea and finally in 500 ml Dialyze Buffer without Urea.
18. The ParC protein was frozen at -20°C.

APPENDIX 20

Preparation of gel column and running FPLC apparatus

1. A syringe filled was filled with dH_2O, the stopper was removed and the syringe was connected to the column.
2. The twist off end was removed and the column was washed with 1 ml dH_2O.
3. The gel column was connected to the apparatus and the UV detector
4. The dialyzed ParC protein was thawed on ice and spun down at 16 000 rpm for 10 minutes at 4ºC, to remove aggregates.
5. The sample was concentrated on a Centricon® Centrifugal filter device and carefully loaded onto the column using a loop. The sample loop had to be full to avoid air getting onto the column.
6. The column was washed with GF buffer. The ParC protein was eluted and collected in fractions according to the recorded peaks on the print.
7. The fractions containing the greatest amounts of purified protein were detected by SDS-PAGE (appendix 17)

APPENDIX 21

Preparation of ParC-CNBr column

1. 300 mg CNBr activated sepharose was washed and swelled in 4 x 15 ml ice-cold 1 mM HCl for at least 30 minutes.
2. The solution was shaken and the supernatant was removed by gentle suction.
3. The resin was washed with 20 ml dH_2O and resuspended in 1 ml dH_2O.
4. The ParC protein (2.5 mg) was diluted in 10 ml coupling buffer and mixed with the CNBr resin.
5. The mixture of protein and gel was shaken at room temperature for 2 hours.
6. The supernatant was removed and unreacted ligards were washed away with 3 x 10 ml coupling buffer.
7. Unreacted sites were blocked with 10 ml 0.2 M Glycin pH 8 (in coupling buffer) for 2 hours at room temperature.
8. The blocking solution was removed away by washing alternately 0.1 M NaAcetate buffer pH 4 containing 0.5 M NaCl and 0.1 M NaAcetate buffer pH 8 containing 0.5 M NaCl.
9. This wash cycle was completed four times.
10. Wash with 50 mM Tris pH 7.
11. If the column was not to be used immediately it was stored in 1 M NaCl/0.01 % NaN_3 at 4°C.

APPENDIX 22

Purification of ParC antibody

1. 1.5 g Protein A Sepharose CL-4B was suspended in dH_2O. The swollen gel was washed for 15 minutes with further 300 ml dH_2O.
2. The gel was poured on the column and equilibrated with binding buffer, 50 mM Tris pH 7.0.
3. 6 ml ParC antiserum was thawed and centrifuged at 13 000 rpm for 10 minutes.
4. The supernatant was poured on the column and the flow-through was collected.
5. The column was washed with 20 ml binding buffer and 4 x 5 ml wash fractions were collected.
6. The column was eluted with 5 ml 0.1 M glycine pH 3.0 and 12 elution fractions were collected.
7. Samples were taken from the fractions and ran on a SDS- polyacrylamide gel and stained with Commassie.
8. The eluted fractions with high IgG content were pooled and neutralized with 1 M Tris pH 9.
9. The ParC-CNBr column was equilibrated with 50 mM Tris pH 7.
10. The IgG solution from step 8 was centrifuged and the supernatant was mixed with the ParC-CNBr resin from appendix 21 and shaken on an end-over-end shaker for 2 hours.
11. The mixture was poured on the column and the flow-through was collected.
12. The column was washed with 20 ml 50 mM Tris pH 7 in 5 ml fractions.
13. The column was eluted with 5 ml 0.1 M Glycine pH 3.0 in 0.5 ml fractions.
14. Samples were taken from the fractions and ran on a SDS-polyacrylamide gel and stained with Commassie.
15. The eluted fractions with high IgG content were pooled and neutralized with 1M Tris pH 9
16. The specificity of the antibody was checked on a Western gel against ParC protein.

APPENDIX 23

Fixation of cells for microscopic studies

1. 2 x 1.5 ml of exponentially growing cells were put on ice.
2. The cells were harvested by centrifugation at 10 000 rpm for 2 minutes at 4°C.
3. The supernatant was removed, and the pellet was resuspended in 1 ml ice-cold, filtered (0.22 μm) TE buffer.
4. The suspension was centrifuged at 10 000 rpm for 2 minutes at 4°C.
5. 100 μl supernatant was left for resuspension of the cells.
6. 1 ml ice-cold, filtered (0.22 μm) 77 % ethanol was added and the sample was stored at 4°C for several months.

APPENDIX 24

Immunostaining

Absorption of unspesific antibody

1. EBO 193 (ΔseqA) cell extract was spun down in order to move aggregates.
2. SeqA antibody was diluted 200 times with cell extract to a final volume of 50 µl.
3. 6 µl PBST with 10 mM EDTA was added and incubated on ice for 1 hour.
4. The supernatant was collected by centrifugation at 15 000 for 15 minutes at 4°C. The supernatant was used as antibody.

Staining fixed cells with antibody

1. An overnight culture was diluted 1:500 in 25 ml desired medium to OD = 0.15.
2. 2 x 1.5 ml of culture was collected by centrifugation at 13 000 rpm for 3 minutes at 4°C.
3. The supernatant was removed and the pellet resuspended in 1 ml in 1 ml ice-cold filtrated (0.22 µm) TE buffer.
4. The suspension was centrifuged at 13 000 rpm for 3 minutes at 4°C.
5. The supernatant was removed except 100 µl which was left for resuspension of the cells.
6. 1 ml ice-cold filtrated (0.22 µm) 77 % ethanol was added and stored at 4°C for several months.
7. A glass slide was cleaned with Decon 90, destilled water and 70 % ethanol. 20 µl 1 mg/ml poly-L-lysine was dropped on the slide, incubated for 5 minutes, washed with dH_2O and air-dried.
8. 10 µl fixed cells were taken from the bottom of the eppendorf tube, added to the glass slide in a thin layer and air-dried.

9. To remove excess of cells the slide was washed with dH_2O and air-dried.
10. The dried area was covered with 100 µl of lysozyme solution and incubated at room temperature for 5-10 minutes.
11. The slide was covered with 1 ml PBST for 30 seconds and inclined to remove the solution. This step was repeated 3 times.
12. The slide was covered with 1 ml 99 % methanol for 1 minute and inclined. The slide was air-dried.
13. 50 µl PBS-T with 2 % BSA was added for 15 minutes to the area for blocking and the glass slide was inclined to remove the solution against a paper.
14. 20 µl antibody (anti-SeqA, see above or ParC from elution fraction 2, see appendix 22 and figure 3.11) was covered with a cover slip and incubated at room temperature for 1 hour in a moisture chamber.
15. The cover slip was removed and the glass slide washed 10 times with 1 ml PBS-T. The remaining solution was removed against a paper.
16. 50 µl PBS-T with 2 % BSA was added for 15 minutes and the glass slide was inclined to remove the solution.
17. 20 µl Cy3-anti rabbit IgG (500 folded) was added; covered with a cover slip and incubated at room temperature in a dark room for 1 hour in a moisture chamber.
18. The cover slip was removed and the glass slide washed 10 times with 1 ml PBS-T. The remaining solution was removed against a paper.
19. 20 µl Mounting medium with antifade for staining of DNA was added and covered with a cover slip.
20. The glass slide was kept at $4^{\circ}C$.

APPENDIX 25

Amino acid and nucleotide sequence of the *parC* gene

(the GenBank accession number is M58408)

(http://www.ebi.ac.uk/cgi-bin/dbfetch?db=embl&id=M58408&style=raw)

```
ID   M58408; SV 1; linear; genomic DNA; STD; PRO; 2284 BP.
AC   M58408; M37832;
DT   17-NOV-1990 (Rel. 25, Created)
DT   17-APR-2005 (Rel. 83, Last updated, Version 4)
DE   E.coli topoisomerase IV subunit (parC) gene, complete cds.
KW   ParC protein; topoisomerase IV subunit.
OS   Escherichia coli
OC   Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales;
OC   Enterobacteriaceae; Escherichia.
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RP   1-2284
RX   DOI; 10.1016/0092-8674(90)90172-B.
RX   PUBMED; 2170028.
RA   Kato J.-i., Nishimura Y., Imamura R., Niki H., Hiraga S., Suzuki
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RL   Cell 63(2):393-404(1990).
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XX

SQ Sequence 2284 BP; 548 A; 571 C; 672 G; 493 T; 0 other;

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