Force generation in dividing E. coli cells: A handles-on approach using optical tweezers

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Chapter 1: Introduction

This thesis is devoted to the development of a novel experimental approach to the study of cell division in bacteria. For this we use the model bacterium *Escherichia coli. E. coli* is a rod-shaped bacterium that lives in the intestines of warm-blooded animals, as well as in many labs around the world. At first sight, it has a not-so-exiting life: it grows by increasing its length, duplicating its DNA and dividing in the middle of the cell by splitting itself in two. However, to actually live this not-so-exciting life is a formidable task for such a small cell, which can duplicate itself within 20 minutes when growth conditions are optimal.

Several complex processes take place within a bacterium; examples are motility, chemotaxis, DNA replication and segregation, transport processes etc. Over the past decades, a lot of the components (genes, proteins, interactions) of these processes have been discovered and characterized. More recently, our knowledge has become sufficiently detailed to start answering questions related to the mechanisms behind the observed phenomena. Advances are both on the biochemical connectivity of the proteins (wiring of the network), as well as on molecular mechanisms that drive or regulate these processes in space and time. Protein complexes that were once thought to consist of static components are now found to consist of highly dynamic subunits.

Cell division is one of the fundamental requirements of all life. It is textbook knowledge that an animal cell divides because a contractile ring of actin filaments and myosin II motor proteins contracts the cytoplasmic membrane ("the purse-string" model (Schroeder 1970; Schroeder 1972). Since animal cells are often thought of as more complex than their prokaryotic counterparts, it might appear surprising that the simple binary fission of a bacterium is still poorly understood. A cell-walled bacterium such as *E. coli* has a three-layered cell envelope, consisting of a cytoplasmic membrane, a thin peptidoglycan (PG) cell wall and an outer membrane (depicted in **Figure 1.1**). It divides by a simultaneous inward growth ("invagination") of all three layers. A simple question such as what drives the growth inwards during division has no satisfying answer. Instead, as in most fields in cell biology at the moment, the list of proteins involved is increasing every month (Errington et al. 2003; Weiss 2004; Goehring and Beckwith 2005; Vicente et al. 2006).

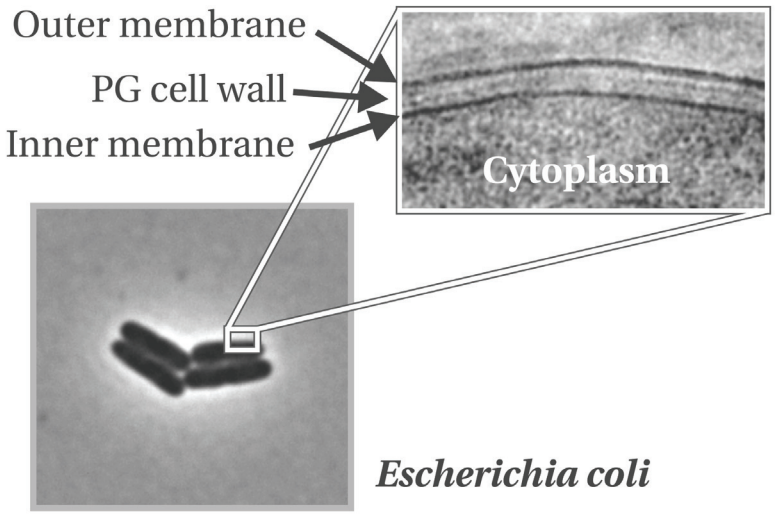

Figure 1.1: ***Escherichia coli.*** A growing micro-colony of *Escherichia coli* cells imaged with a phase-contrast microscope. After zooming in on the cell envelope (inset), a Cryo EM picture shows the crowded (electron dense) cytoplasm, surround by three layers: the cytoplasmic membrane, the peptidoglycan (PG) cell wall and the outer membrane. The compartment delineated by the inner and outer membrane (containing the PG cell wall) is called the periplasm. (Cryo EM picture reproduced from (Matias et al. 2003))

However, upon closer inspection of the animal cytokinesis field, both fields appear to be in a quite similar stage: It too, is faced with long "parts lists", together with rudimentary models that basically cover the entire imaginable spectrum (Eggert et al. 2006). After discussing the textbook "purse-string" model and two other models, the authors conclude with the following remark: "*In our view, the mechanics of cytoskeletal force production in cytokinesis is still an open question"* (Eggert et al. 2006).

Until the early to mid 1990's, bacteria were viewed as "bags of enzymes" that function as a result of simple physical principles such as tension, pressure and macromolecular crowding (Koch 1988). This made them distinct from eukaryotic cells, where observed phenomena were already being interpreted within a context of cytoskeletal elements such as microtubules and actin filaments, which together with motor proteins such as kinesin and myosin are responsible for mayor cellular events such as chromosome segregation

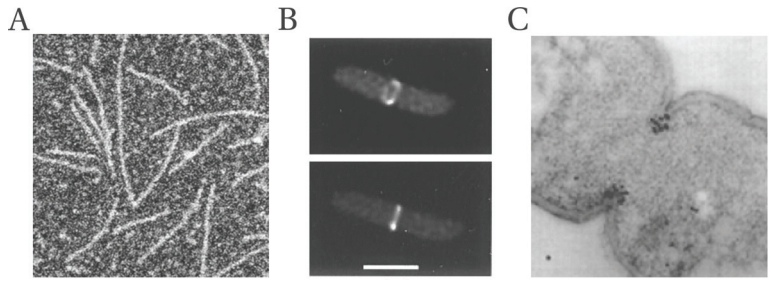

Figure 1.2: The Z-ring. (A) FtsZ polymerizes in vitro into protofilaments. (Reproduced from (Romberg et al. 2001)). Thickness of a protofilament is ~5 nm. **(B)** FtsZ forms a ring at mid-cell *in vivo* (Z-ring). Deconvoluted fluorescence microscopy images of cells expressing FtsZ-GFP. Bar indicates 1 μm (reproduced from (Ma et al. 1996)) **(C)** Division in *Escherichia coli* occurs through the simultaneous invagination of the three layers that constitute the cell envelope: Inner membrane, PG cell wall and outer membrane. Shown is an EM picture of a dividing *Escherichia coli* bacterium. The black dots are anti-FtsZ gold nanoparticles. (Reproduced from (Bi and Lutkenhaus 1991))

and cell division (Alberts 2002).

Recent years have seen these distinctions to fade, as more and more cytoskeletal elements are being discovered in bacteria (Cabeen and Jacobs-Wagner 2005). Of these, the actin homologue MreB and the tubulin homologue FtsZ have made the biggest impact on the field. MreB is found to polymerize into helical cables throughout the cell underneath the cytoplasmic membrane and is important for its rod-like shape (Jones et al. 2001). FtsZ polymerizes into filaments (**Figure 1.2A**) that form a ring in the middle of the cell (**Figure 1.2B,C**) and is required for formation of new spherical caps during division (Bi and Lutkenhaus 1991).

These new findings suggest that cytoskeletal elements “shape” the cell. Evidence is accumulating that FtsZ forms a force-generating contractile ring which directs cell wall growth inwards during division (Osawa et al. 2008). Likewise, the prevailing view of cylindrical cell wall growth is now based on cell wall synthesizing machinery that is guided along dynamic helical MreB tracks (den Blaauwen et al. 2008). It is possible that the maintenance of the rod-shape also requires inward forces to counter the turgor pressure and membrane tension (see also **Chapter 2**).

However, although appealing and likely, direct experimental evidence for a force-

induced cell shaping during growth and division is lacking. In this thesis, we describe the first steps towards a novel approach to study the role of forces generated by cytoskeletal elements inside living bacterial cells. The approach is based on the creation of artificial anchoring points on the cell surface at the site of division. This allows the attachment of sensitive force probes (optically trapped beads) that can locally exert and measure force simultaneously (**Figure 1.4A**). As optical tweezers allow easy manipulation of multiple trapped beads in a liquid environment, together with light microscopy, this approach has the potential of measuring the effect of external forces on the division process of a single cell.

Since to date in bacteria, no (protein) markers have been found that are localized at the site of division which are accessible from the exterior, such a protein has to be created first. A daunting task already by itself, ill-characterized bacteria, or ones that do not have the proper genetic tools available cannot be used. This leaves the main bacterial model organisms *E. coli* and *B. subtilis*. We chose *E. coli* because *B. subtilis* has a thick ~20 nm cell wall, and forms a proper cell wall septum (a double layered separation plate in the middle of the cell), which is only cleaved after its formation is complete (Fukushima et al. 2006). This means that the presumed force-generating process, the Z-ring contraction during septum formation, is shielded by a thick cell wall from the outside world.

In contrast, the cell envelope of *E. coli* consists of a cytoplasmic membrane, surrounded by a thin ~5 nm thick PG cell wall, in turn surrounded by the outer membrane (Matias et al. 2003) (**Figure 1.1**). The cellular compartment formed between the inner and outer membranes is called the periplasm (solvent accessible width ~15-20 nm (Matias et al. 2003). Since in *Escherichia coli* the envelope layers invaginate simultaneously (**Figure 1.2C**), and the FtsZ ring is expected to exert small forces on the growing cell wall, this makes it conceivable that small forces (below 100 pN) exerted on the cell wall via an artificial construct in the OM can influence constriction.

Measuring and exerting forces with optical tweezers

Optical tweezers are capable of exerting and measuring forces typically in the range of ~0.1-100 pN (Moffitt et al. 2008). The use of high refractive index particles holds a promise to further increase the maximum force (van der Horst et al. 2008). These forces are typically those encountered inside living cells. As such, the technique is used extensively to study biological processes such as protein folding and force generation by molecular

motors and biopolymers (Visscher et al. 1999; Kerssemakers et al. 2003). This is accomplished by optical trapping of a micron-sized, spherical, transparent particle (a "bead") in a highly focused laser beam. Physically, this is possible because light carries momentum $\vec{p}$ with magnitude $p = E/c$ (with E the energy of the photons and c the speed of light) and direction $\vec{k}$ (wave vector). When photons change direction due to refraction by the particle, this momentum vector is changed, implying that a force has been exerted on the light wave ($\vec{F} = \frac{d\vec{p}}{dt}$) (Newton's second law). Since the particle has exerted a force on the light wave, it follows that (from Newton's third law) that the light exerts an equal but opposite force on the particle (Svoboda and Block 1994).

Neglecting absorption, the forces exerted on the particle are caused by refraction and reflection of light. Typically, these forces are split into the gradient force that is directed in the direction of the light gradient (i.e. the laser focus) and the scattering force, that is directed along the optical axis and pushes the particle out of the focus. In 1986, it was demonstrated by Arthur Ashkin that these forces can compensate each other and stable three-dimensional (3D) trapping of micron-sized particles is possible (Ashkin et al. 1986).

How this is possible can be understood from a simplified ray-optics picture (Svoboda and Block 1994). In **Figure 1.3A**, the brighter ray 2 causes a larger reaction force than ray 1, and thus the resultant force points in the direction of increasing light intensity, i.e. towards the focus. The scattering force is along the optical axis, in the direction of the light, and pushes the particle out of the focus. In **Figure 1.3B**, it is shown that just below the focus, highly divergent rays can enter the particle to become less divergent. This creates a restoring force towards the focus. At the location where the scattering force balances this restoring gradient force stable 3D trapping occurs.

When an external force F pulls the trapped particle out of the trap, it responds (for small displacements x) as if it is attached to a harmonic spring, $F = kx$. When the spring constant k, also called the trap stiffness, is known, measuring the displacement of the particle becomes equivalent to measuring the force on it. The bead has become a sensitive force probe. Measuring the Brownian motion of the particle in the trap allows the determination of the trap stiffness (see also **Chapter 6**) (Visscher et al. 1996).

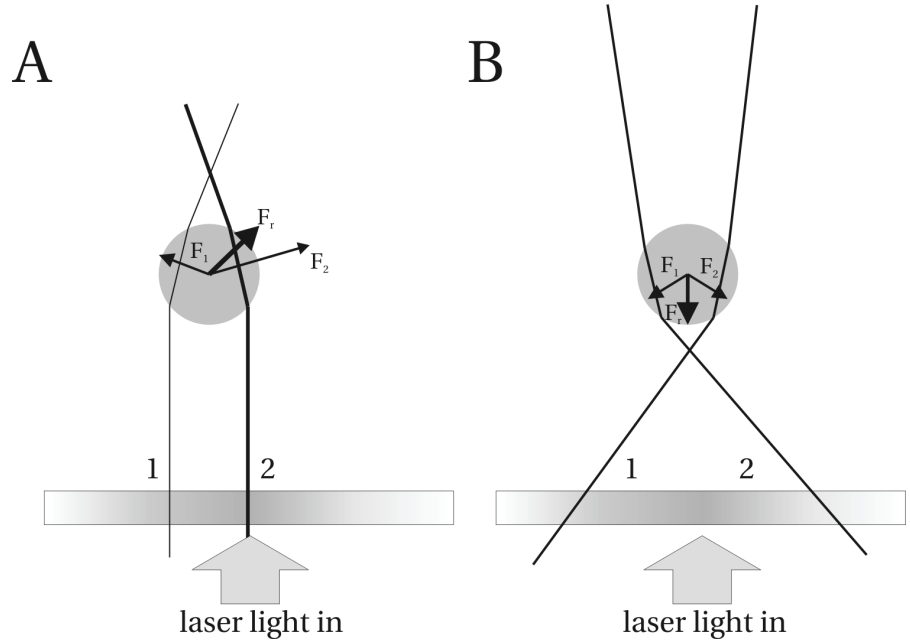

Figure 1.3: Stable 3D optical trapping of a transparent particle ("bead"). Individual light rays are drawn, the intensity gradient is indicated in the gray bar. (A) The bead is attracted to the region of highest laser intensity. (B) "Behind" the focus, stable 3D trapping can occur.

Force probe attachment to the site of division

For the study of FtsZ force generation, ideally one wants to measure and exert forces on the same entity on which FtsZ supposedly acts, i.e. the cytoplasmic membrane. As it is not possible to "strip" a bacterium of its OM and PG cell wall without losing its ability to divide, a molecular spacer construct that bridges the periplasm is required. In doing so, we assume that the multitude of molecular bonds that link the three layers together will efficiently transduce the force between Z-ring and trapped bead "handle".

To create an anchoring point on the cell surface, we chose to genetically insert an antigenic peptide (epitope) into the surface-exposed loops of the highly abundant OM β-barrel protein OmpA (Freudl 1989). As the inward growth during division of the bacterium is relatively sharp, the bead handle cannot be directly attached to the division site, and a spacer is needed. We use dsDNA, as its force-extension behavior is well known (Smith et al. 1996), and its ends can easily be functionalized for attachment to either a bead, or to a

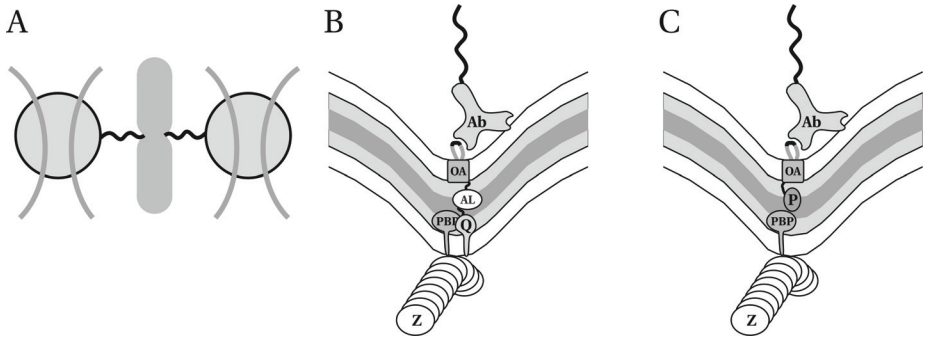

Figure 1.4: A molecular construct to bridge the periplasm. (A) Proposed experimental geometry. A bacterium is tethered via dsDNA to optically trapped beads. (B) Molecular construct based on mid-cell affinity protein FtsQ. (C) Molecular construct based on mid-cell affinity protein Pal. (B,C) Protein abbreviations: Z-ring (Z), the PG synthesizing complexes (PBP), FtsQ (Q), ALBP (AL), OmpA β-barrel (OA), Antibody (Ab), Pal (P).

protein such as an antibody or streptavidin (Cecconi et al. 2008).

Protein domain fusions to OmpA are employed to localize the OmpA β-barrel to mid-cell (**Figure 1.4B,C**). By fusing the OmpA domain to a protein domain that has mid-cell affinity (either for another mid-cell localized protein, or for a particular substrate present at mid-cell), we aim to localize the OmpA protein to mid-cell.

The location of the native cellular compartment of the mid-cell affinity domain determines the length and complexity of the resulting fusion protein. When this research was initiated (2002), no periplasmic or outer membrane mid-cell affinity domains had been discovered yet. Therefore, the inner membrane cell division protein FtsQ (van den Ent et al. 2008) was chosen as mid-cell localizing domain, fused via a spacer domain (in **Figure 1.4B** indicated with the ALBP sugar binding protein domain) to bridge the periplasm to the OmpA β-barrel.

With the discovery of the Pal protein at mid-cell (Gerding et al. 2007), an OM lipoprotein that has mid-cell affinity and binds PG became available, and a much simpler construct became possible (**Figure 1.4C**), as the Pal domain can be directly linked to the OmpA β-barrel.

Outline of this thesis

Since the force-induced cell shaping idea is relatively new, in **Chapter 2**, we will review the experimental evidence on which it is based, and the various mechanisms proposed that shape cell-walled organisms.

The remaining part of this thesis is divided in two parts. The first part deals with our quest for a fusion protein that is accessible on the cell surface and localizes predominantly to the division site: In **Chapter 3**, we describe the construction and characterization of engineered OmpA proteins with various epitopes. The OmpA variants were specifically tested for efficient membrane insertion *in vivo*. An epitope insertion fusion was found that inserted as efficient as wild-type OmpA. In **Chapter 4**, the construction of fusion proteins based on mid-cell localization domain FtsQ are described, and characterized for their ability to localize to mid-cell. After the discovery of a mid-cell localizing domain (Pal) that is tethered to the OM at its periplasmic side, as well as a fluorescent protein (mCherry) that fluoresces in the periplasm, we fused these domains to the OmpA β-barrel and characterized the resulting fusions for their ability to localize to mid-cell and/or to insert in the OM (**Chapter 5**).

The second part deals with the experimental setup (**Chapter 6**) used for the optical tweezers experiments described in **Chapter 7**. In these experiments, cells are immobilized on a surface, and DNA-coated beads are attached specifically to the engineered OmpA anchoring point on the cell surface. Force-extension curves of DNA tethers to the OmpA β-barrel are compared to the full-length OmpA that contains a cell wall binding domain. The data contain evidence for an increased probability of membrane tube formation when OmpA is not attached to the PG cell wall. The thesis concludes with a chapter that summarizes how far we have come, discusses alternative approaches, and provides recommendations for further experiments (**Chapter 8**).