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

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VIII

Chapter 8: Final Considerations and Recommendations

This chapter will discuss the steps remaining before one can actually exert and measure forces during the division of a single bacterium. We present alternative approaches that we explored, as well as provide recommendations/possibilities for future research. However, we start by summarizing what has been achieved so far:

First, we have engineered a protein (the OmpA β-barrel with the streptavidin-binding peptide) that inserts as efficiently into the outer membrane as wild-type OmpA (**Chapter 3**). Second, we have shown that using this protein, it is possible to attach optically trapped beads to the cell surface of a living bacterium, and we have estimated that a single tether can withstand forces of a few pN for a few minutes (**Chapter 7**).

Major effort has been directed towards the construction of a β-barrel fusion protein that localizes at mid-cell (**Chapter 4 and 5**). A lot has been learned, and as discussed below, we believe that such a fusion protein is technically possible and feasible. To obtain such a fusion protein, two approaches are explored in this thesis: First, the dynamic association of either an endogenous inner membrane protein (e.g. FtsQ) with the "divisome" at mid-cell, followed by OM insertion of its periplasmic "tail" containing the β-barrel protein (**Chapter 4**). Second, the dynamic association of an endogenous periplasmic factor (e.g. Pal) at mid-cell while anchored to a β-barrel present in the OM (**Chapter 5**). The first approach requires the sequential steps of mid-cell association of e.g. FtsQ followed by passage through the septum and OM insertion of the β-barrel. The second approach requires the free diffusion of the β-barrel through the OM as well as that the periplasmic factor must be able to bind to its "septal partner"/molecule/protein at mid-cell when attached to the β-barrel.

Below, we present an overview of the literature on the lateral mobility of β-barrels in the OM of *E. coli*. Although sparse, the current experimental evidence available suggests that free diffusion of β-barrels is indeed possible, especially in *E. coli* strains that produce a truncated LPS, such as K-12. Based on the results obtained in **Chapter 5**, we then provide some suggestions for periplasmic factors on which future fusion proteins could be based.

In parallel with the approach as outlined in the introduction, we have explored

alternative approaches. One approach to avoid the engineering of a mid-cell localized anchoring point in the OM is to make use of a so-called "L-form" *E. coli* strain. This is a mutant strain that has lost irreversibly the capacity to form an OM and PG cell wall. Our (limited) experience with such a strain will be discussed.

In the original experimental concept, the bacterium is attached to optically trapped beads tethered to it on both sides. This fixes the bacterium in space at the point where both forces exerted on it are equal but opposite. Without a specific anchoring point at mid-cell, it is difficult to imagine how two DNA-coated beads can be attached to the division site of a single bacterium on opposite sites. However, it might be possible that after bringing a DNA-coated bead into contact with the bacterium, the bead will be attached roughly at the division site. Then, in the absence of an opposing force by a second tethered bead, an alternative way of holding the growing bacterium in place is required. We have explored two possibilities: the surface approach, and the "dumbbell" approach. The first approach has been the topic of **Chapter 7**, and results that we obtained with the second approach are presented in this chapter.

Finally, we discuss a promising way to improve the strength of the weakest part of the molecular construct, the attachment of the free end of the DNA to the β-barrel. We conclude by discussing two experimental approaches to study the effect of forces on bacterial (wall) growth and shaping in a more general way.

Mobility of β-barrels in the OM

The OmpA-(mid-cell domain) fusion approach relies heavily on the assumption that the OM is a continuous fluid bilayer and that the OmpA β-barrel fusion protein can associate dynamically with periplasmic factors when the cell divides.

Traditionally, the OM is thought of as a tight, gel-like barrier with LPS packed together with cations in a crystalline matrix (Nikaido 2003; Ruiz et al. 2006). Only a hand-full papers exist that study or provide clues on the molecular organization of the bacterial outer membrane and the lateral mobility of its constituents.

Chemically reactive fluorescent dyes exist that are thought to label only surface-exposed cell components. Using such a dye (Texas Red-X-succinimidyl ester), all cell surface components that contain reactive amine-groups can be fluorescently labeled. From pulse-chase and FRAP experiments on labeled *E. coli* cells, it was concluded that there are mobile and non-mobile "elements" in the OM (de Pedro et al. 2004; Ghosh and

Young 2005). However, it remains unclear which components exactly were labeled in these studies. Periplasmic lipoprotein Lpp was also found to become labeled (de Pedro et al. 2004), and reactive agents based on succinimidyl ester have been shown to label also periplasmic and inner membrane components (Bradburne et al. 1993; Sabarth et al. 2002).

Specific diffusion studies have focused on the β-barrel LamB which forms trimers in the OM. LamB in the OM of a living bacterium with a 540 nm streptavidin bead attached was shown to be confined within 25 nm and have a diffusion coefficient of 0.15 $\mu m^2/s$ (Oddershede et al. 2002). A different study was reported that used either fluorescent phage tails or ~20 nm gold beads to track LamB proteins in living cells (Gibbs et al. 2004). Surprisingly, large differences in localization patterns between fixed and living cells were observed for LamB. Tracking 20 nm gold beads attached to LamB in living cells, it was found that two sub-populations existed, termed "mobile" LamB (40% of all trajectories, bead moved over 300 nm in 5 min) and "immobile" LamB (60% of all trajectories, bead moved over 50 nm in 5 min). To explain the immobile LamB, both studies refer to an early observation that LamB interacts with PG (Gabay and Yasunaka 1980). However, from the crystal structure it is not clear how PG binding would be achieved (Schirmer et al. 1995). Possibly, a periplasmic factor could retain LamB.

Indirect evidence for lateral diffusion of β-barrels in the OM comes from studies of the IcsA virulence protein in *Shigella* that forms actin-tails, which propel the bacteria after infection in eukaryotic cells. The IcsA protein is part of the autotransporter family of OM proteins that are large proteins consisting of a cell surface-exposed N-terminal domain anchored in the OM by a C-terminal β-barrel (Bernstein 2007). Autotransporters are sub-localized to old poles already in the cytoplasm by an unknown mechanism (Jain et al. 2006). At the pole, they are exported to the periplasm by the Sec system (which itself is not specifically localized at the pole) and the β–barrels are inserted in the OM mediated by Omp85/BamA (Jain and Goldberg 2007). After polar OM insertion, IcsA laterally diffuses through the OM, forming a concentration gradient, as observed with immunofluorescence (Robbins et al. 2001).

Additionally, there are indications that LPS composition can influence the lateral mobility of β-barrels. Intact LPS molecules are thought not to diffuse, based on a FRAP experiment using fluorescent concanavalin A labeling of *E. coli* with intact LPS (Ghosh and Young 2005). Mutations in LPS synthesis genes affected polar localization of IcsA in the

OM (Sandlin et al. 1995). Based on this, interactions with LPS were proposed to retain the β-barrels at the cell pole (Jain et al. 2006). Consistent with this hypothesis, polar localization of IcsA is reduced in K-12 strains that have truncated LPS, possibly due to increased lateral diffusion (Jain et al. 2006).

Finally, for a different OM β-barrel (the BtuB protein) a diffusion coefficient similar to that of the IM was recently reported (Jeff Spector and Ken Ritchie, Biophysical Society Meeting 2007). Thus, the available evidence suggests that β-barrels *can* diffuse freely in the OM.

It will be important to find out whether this also holds true for the OmpA β-barrel. FRAP experiments on living cells with fluorescent probes attached to the surface-exposed loop might answer this question. Especially the SA-1 peptide is useful for this approach, as this peptide can be directly visualized with fluorescent streptavidin. Although streptavidin is a tetramer, steric hindrance will likely cause it to bind only one OmpA, possibly two. Alternatively, fluorescently labeled anti-FLAG can be used to visualize the FLAG-containing OmpA β-barrel. As a control, full-length OmpA can be used, which we expect to be immobilized or slowed down by its interaction with the PG cell wall.

Restraining a β-barrel to mid-cell

Based on the results with LamB, BtuB and IcsA, it is likely that OmpA also can diffuse in the OM. This would satisfy one of the requirements of the approach followed in **Chapter 5**, which is the fusion of periplasmic factors to the OmpA protein to sub-localize it to the division site. The alternative approach, extending an IM protein, such as FtsQ (see **Chapter 3**), requires that the fusion protein somehow can pass through the PG cell wall without interfering with the dynamic association of the IM protein at mid-cell, for instance, when turnover in the cell wall is so high that it can diffuse laterally while spanning the periplasm. If PG turnover is low, then the timescales of mid-cell association and OM insertion of the periplasmic "tail" should be sufficiently separated, with mid-cell localization occurring first, followed by OM insertion of the tail. In this scenario, an additional worry is what happens after division, i.e. whether the fusion proteins are subsequently "trapped" at the new poles. This would put constraints on synthesis and folding rates of the fusion, as well as create possible problems with titration of "divisome" factors away from mid-cell.

In **Chapter 5**, various OmpA constructs are described that were designed to localize to

mid-cell. Of these, Pal was highly promising due to its homology to the OmpA PG binding domain and its sub-cellular localization (between PG and OM). After fusing Pal to the C-terminus of OmpA, it appears that Pal can no longer compete with wild-type lipoPal. In the absence of wild-type lipoPal, OmpA-Pal was not functional. As only the OM anchoring has changed, possibly the rapid diffusion of Pal in the inner leaflet of the OM is important. After fusing OmpA to the C-terminus of Pal, OmpA can no longer insert into the OM.

Thus, an alternative domain is needed. From the results obtained with the OmpA-Pal fusion, we can conclude that a periplasmic factor is preferred that normally localizes to the space between the PG cell wall and OM at mid-cell and has no strong phenotypic defects when non-functional.

AmiC, a PG hydrolase that localizes at mid-cell and helps to cleave the septum (Heidrich et al. 2001; Bernhardt and de Boer 2003), appears to match these requirements. The catalytic function and its septal localization are separated into two domains, as without catalytic domain the septal domain ^{T}AmiC localizes to mid-cell. In **Chapter 5**, only a C-terminal fusion is presented, as initially we reasoned that since ^{T}AmiC localized with a C-terminal GFP, this terminus could accept a fusion without destroying mid-cell localization. However, this domain order prevented OM insertion of OmpA. Possibly, fusing ^{T}AmiC to the C-terminus of OmpA allows OM insertion and mid-cell localization at the same time. If competition with wild-type AmiC occurs, a ΔAmiC strain can be used. In (Heidrich et al. 2001), it is shown that a ΔAmiC strain growing in LB medium at 37ºC forms chains of 3-6 cells in 20-30% of all the cells. Chaining was most obvious in late stationary phase. Thus, in 60-70% of the cells, septum cleavage still occurs, although possibly at a reduced rate.

If AmiC fails to perform, other proteins might take its place. For example, it was recently discovered that the outer lipoprotein RlpA localizes to mid-cell ("rare lipoprotein A", DS Weiss lab). It has a C-terminal "SPOR" domain that binds PG. Deleting it has apparently no effect on cell division (DS Weiss, Gordon Conference 2008). Another outer lipoprotein is NlpI, which is thought to function during cell division but has not been localized to mid-cell yet (Ohara et al. 1999). This protein is less attractive however, since deleting it renders cells osmotically sensitive (Ohara et al. 1999), which could be indicative of OM problems during division similar to Pal.

Alternative approach: PG cell wall-less *E. coli* ("L-forms")

As an alternative approach, we have considered *E. coli* mutant cells without PG cell wall and OM (so-called L-Forms) as an alternative. Here we describe what is known, as well as our experience with it.

Treating *E. coli* with lysozyme/EDTA is a standard procedure to destroy its cell wall and permeabilize its OM (Neu and Heppel 1964; Harvey et al. 2004). After such treatment, it has been reported that proteins up to 240 kDa can enter the periplasmic space (Harvey et al. 2004). However, such treatment typically abolishes division, and does not strip the cell entirely of its OM. In the literature, stable PG cell wall-less *E. coli* have been reported. They come in two variants: Spheroplasts are cells that contain IM and OM, but no PG. Protoplasts are cells that contain only IM. One such protoplast strain (as judged from EM pictures (Gumpert et al. 1971)), LW1655F+, was created by continued passages in complex medium in the presence of 6-10% sucrose and penicillin. After some time, this strain lost the ability to grow a cell wall in the absence of penicillin ("revert"), and became a stable "L-form" strain.

Obviously, a strain that contains a Z-ring, but no PG cell wall and OM would be very useful in our proposed optical tweezers experiment, as there is no longer a requirement for an OM fusion protein that artificially localizes to mid-cell. In principle, simply adding an epitope to a (now surface-exposed) periplasmic domain of one the known cell division proteins (such as FtsQ or FtsN) would be enough.

However, there are several drawbacks: It is osmotically sensitive, requires growth in complex media (e.g. beef-heart-infusion and yeast extract medium) and large inocula (a dense culture is typically diluted 10-100x during a passage to fresh medium) (data not shown, (Siddiqui et al. 2006)). A growing culture is quite heterogeneous: cell size ranges from a few hundred nm to a few μm in diameter (data not shown, (Schuhmann and Taubeneck 1969)). The growth rate is slow, probably partly because division often fails (lysed cells accumulate in the medium). This is probably why a large starting inoculum is needed.

While we were performing preliminary growth experiments with the LW1655F+ strain, a work came out (Joseleau-Petit et al. 2007) that described a method to turn any *E. coli* strain into a "L-form-like" state (envelope structure: both IM and OM in "somewhat disorganized state" (Joseleau-Petit et al. 2007)). Using this method, the authors showed

that their L-form-like cells contained residual PG (~7%), and that PG synthesis was required for their propagation. According to the authors, no published data exists which excludes that in the LW1655F+ L-form, also PG synthesis takes places during division. This might interfere with epitope exposure by periplasmic domains of cell division proteins, as the septal PG covers them during division. However, differences might exist between LW1655F+ and the L-form-like cells. The latter cells appear to have both IM and OM, whereas EM pictures of LW1655F+ showed a single membrane. Furthermore, without a periplasmic space and an OM, it is unclear how septal splitting would occur, as PG hydrolases such as AmiA, AmiB and AmiC are thought to diffuse freely in the periplasm. Without a periplasm, they would be expected to leak into the external medium.

Due to the fact that it was not clearly established that our L-form protoplast indeed did not contain septal PG, L-form division is slow and often fails, and the fragility of the cells (demonstrating epitope exposure specifically at mid-cell requires labeling of living cells) this option was considered too risky and put on hold.

Experimental geometry: assembly of the construct in the trap

In **Figure 8.1**, schematics and preliminary experiments are shown that demonstrate the assembly of a bacterium tethered to one or two DNA-coated beads. In these experiments, the DNA tethers are formed during the experiment in a controlled manner. Alternatively, DNA-beads and cells can be mixed and pre-incubated. However, it is anticipated that after finding a floating bacterium with two DNA-coated beads attached, it will be difficult to make sure that the two beads end up in two separate traps with the bacterium in between.

Note that for these experiments we switched to defined medium, as the commonly used LB/TY medium appeared to interfere with the formation of biotin-streptavidin bonds during DNA tether formation. In retrospect this was not so surprising, since LB/TY medium contains yeast extract, which is a rich source for B vitamins such as biotin (vitamin B7). Presumably, the free biotin binds to streptavidin at the ends of the DNA-coated beads, blocking tether formation to the biotinylated anti-FLAG on the cell surface.

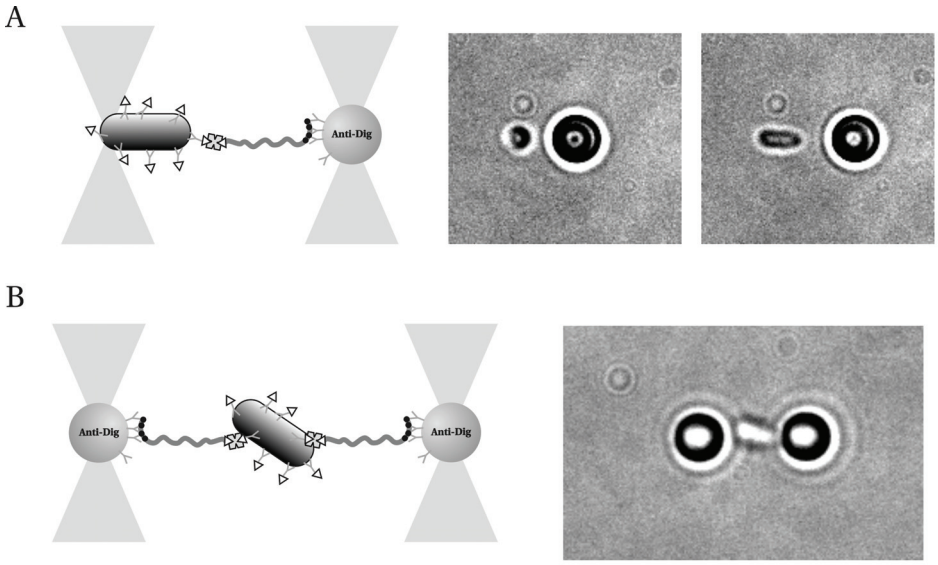


Figure 8.1. Experimental geometries using DNA spacers. (A) A DNA tether is formed by trapping both an antibody-coated bacterium and a DNA-coated bead and forming a biotin-streptavidin bond after bringing both cell and bead in close proximity using two optical traps. (Here the two traps originated from the two opposed objectives and were manipulated manually by XYZ telescopes, i.e. the traps are not time-shared). **(B)** Using dynamic time-shared traps, first a DNA tether was established as described in (A), after which a second DNA-coated bead was brought into close proximity to the trapped and tethered bacterium. After formation of the second DNA tether, the trap holding the bacterium is turned off and the bacterium is held via two DNA spacers in double optical traps. Here, after a few seconds, the second tether broke and the bacterium was pulled towards the trap holding the bead it remained tethered to.

As the experiment is expected to take several minutes, drift, and unwanted objects jumping into the trap can be a problem. Using rich medium (such as the defined Rich medium used in **Chapter 7**) results in fast growing cells, so that the division occurs as rapidly as possible. Drift of the stage with respect to the optical trap is ~ 1 nm/s at room temperature. By using two time-shared traps directed into the sample via one objective, almost no drift is expected to occur between the two traps, and the residual drift will most likely not be dependent on the sample temperature.

Effect of laser light on bacterial growth

As mentioned, a controlled assembly of the construct is preferred. This requires directly trapping a bacterium using optical tweezers. As the bacterium must divide after assembly, photo-damage to the cell must be minimized.

Soon after the invention of single-beam optical tweezers, it was found that infrared (IR) laser light is much less damaging to cells compared to visible laser light from a green argon laser, which could even "opticute" (optically execute, shrink) *E. coli* cells (Ashkin et al. 1986). Damage-free trapping of *Escherichia coli* using 80 mW IR laser light for 30 min was reported (Ashkin et al. 1987). However, (Neuman et al. 1999) et al. monitored flagellar rotation rate as a measured of cell viability, and found that using 1064 nm light, within a few minutes rotation rate decreased when trapped with 100 mW in the specimen.

The cells can be briefly trapped without loss of viability: (Ericsson et al. 2000) reported an assay where *E. coli* cells were sorted with optical tweezers, and demonstrated viability by monitoring subsequent growth following the trapping event. Furthermore, (Roos 2000) also showed that an *E. coli* bacterium could grow (slowly) when trapped in weak traps at high-growth speed/rich medium conditions.

In a systematic study of 1064 nm optical trapping damage on trapped *Escherichia coli* (Ayano et al. 2006), the ability to divide was checked after trapping at various times and laser intensities. It was found that the total energy (Power x time) was the determining factor in the loss of division capability. At energy < 0.36 J, division was not affected. For a laser power of 10 mW (measured at the objective), this means 36 s of trapping time before division is affected! Furthermore, division was more easily affected than growth. Recently (Rasmussen et al. 2008), it was shown that continuous IR trapping of *Escherichia coli* at 18 mW in the sample decreases the pH gradient within minutes. Overall it can be concluded that cells can be trapped without loss of viability, but that both trapping time and laser power need to be minimized.

Using time-shared tweezers, we trapped several bacteria at both poles (rod-shaped cells align along the optical axis when trapped) in minimal medium with the water bath temperature at 40°C to monitor length growth. Under these conditions, cells on agar would readily grow into micro-colonies. However, we have never observed growth when directly trapping a bacterium. It is possible that the light intensity in the traps was too high, or that in minimal medium, less light is required to damage the cells (the cells are

also smaller).

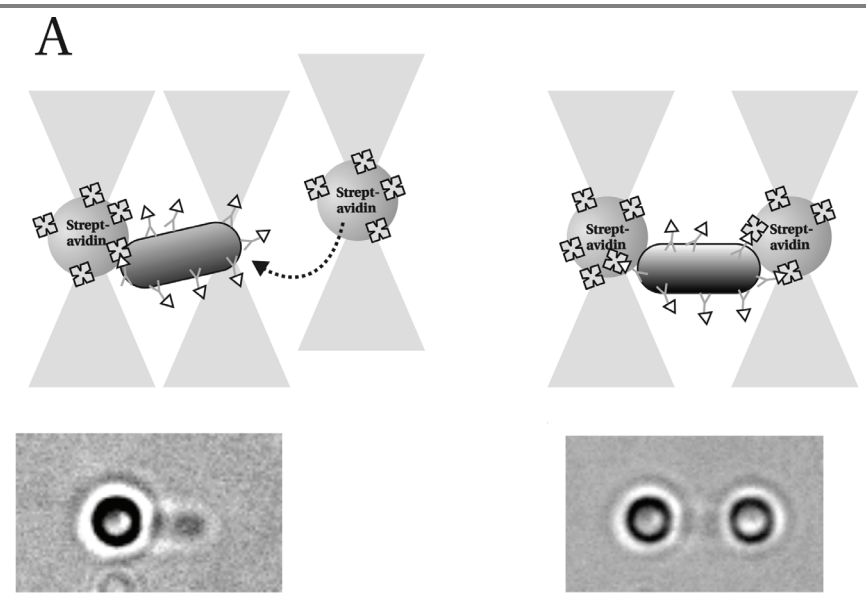
Alternative geometries: surface and dumbbell approach

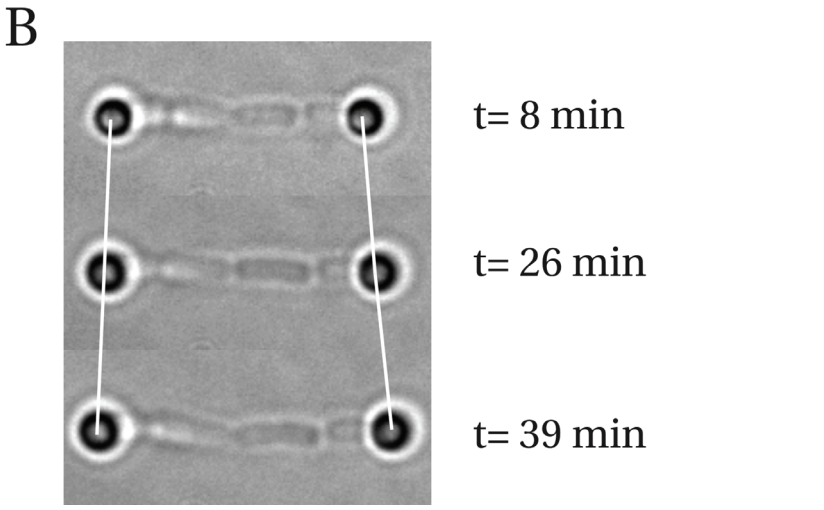
Thus, we concluded that during the experiment, which takes at least several minutes, direct laser trapping of the cells is not an option. We therefore looked for ways to hold or immobilize a bacterium without directly trapping it, to prevent a block in growth and/or division. We have explored two options: The first ("surface approach") is described in **Chapter 7**, where tethers are formed on immobilized bacteria on a glass surface (adhered aspecifically). The second ("Dumbbell approach) is discussed below, and consists of attaching beads as handles directly to the cellular poles.

The disadvantage of the surface approach is that most bacteria are not attached strong enough to withstand forces > ~5 pN for more than several seconds. A second disadvantage is the three-dimensional geometry of the experiment. Not only lateral, but also axial displacement of the trapped bead has to be taken into account (**Chapter 7**). This can be avoided by sticking bacteria on beads immobilized on a cover slip (Fallman et al. 2004; Jass et al. 2004): this increases the height of the bacterium such that the tether can be made "from the side" in the XY plane. A disadvantage is that refraction of light through the bead (partially) obscures the bacterium. This might not be a problem when measuring e.g. the elasticity of pili or flagella, but it is difficult to see if growth occurs, or if the bacterium displaces (data not shown). In general, the surface approach tries to reconcile two conflicting requirements: to immobilize the bacterium as strongly as possible on the one hand, while on the other hand allowing free unconstrained growth and division.

Therefore, we have experimented with alternative geometries where beads are attached to the bacterial cell poles, and the cell is held by optically trapping the bead handles (**Figure 8.2**). This approach was pioneered in (Roos 2000), where bacteria were mixed with poly-L-lysine coated (core-shell/silica) beads (d = 675 nm) and a bacterium with two beads was trapped in two weak traps (5 pN/μm, 13 mW per trap estimated in the sample) and followed in time. It was found that in rich TY medium at 37°C, the bacterium could continue to grow and divide when held via these handles.

We followed a similar approach. To attach beads we use our specific OmpA-FLAG anchoring (see also **Chapter 3**). The connection between bead and cell is through biotinylated antibodies and streptavidin-coated beads (**Figure 8.2**). Preliminary experiments in bulk indicate that bacteria can grow normally after antibody labeling (after





labeling a lag phase of ~30 min was observed when grown in shakers and following the

Figure 8.2. Growing cells in the dumbbell geometry. (A) Assembling the dumbbell. Three time-shared traps were used to create the dumbbell bacterium trapped in between two beads. First, a bacterium is trapped in two traps (not shown), then with the third trap, a streptavidin bead is pressed to one of its poles and the trap holding that pole is shut off. This is the situation shown in the left panel. Then a second streptavidin bead is attached to the other pole, and then the trap holding that pole is shut off too. The result is shown in the right panel. **(B) Growth within the dumbbell.** After assembling a chain of four bacteria (held together presumably by anti-FLAG antibodies) into dumbbell geometry, the two cells close to the IR laser traps do not grow, whereas the two cells suspended in between grow normally. **Experimental protocol**: LMC500 cells expressing (OmpA-177)-FLAG were grown in GB1 minimal medium at 28°C in the presence of antibiotics and 30 μM IPTG (continuous induction). In the morning, cells were diluted in fresh medium and grown to OD450~0.2. Then, cells were blocked with 0.1% BSA in GB1 medium and labeled with biotinylated anti-FLAG (M2) antibody (e.c. 50 μg/ml). After washing with medium the cells were mixed with 1.26 μm diameter streptavidin-coated PS beads (Spherotech, USA) and flown into a sample cell. The sample cell consisted of #1 cover slips (circular, diameter 22 and 15 mm) dipped in melted agarose (0.4%) and dried in a pincer. A flow cell was created by drawing two grease lines on the 22 mm cover slip placed in the sample holder and pressing the 15 mm cover slip on top. The sample was sealed with candle wax. This allows prolonged measurements at elevated temperatures. The water bath was set at 40°C, which results in a sample temperature of ~30°C.

optical density). However, in the trap, after labeling with anti-FLAG, cells grew as chains. Possibly, anti-FLAG binds to FLAG epitopes on both cells, preventing the cells from separating. Chains could be split into individual cells by employing high-powered laser traps (as cells are trapped less efficiently as beads, we estimated forces < 10 pN). Unfortunately, we never observed any growth in any of the dumbbell bacteria. We estimate that the trap stiffness of the polar bead handles was typically a few tens of pN/μm. Again, too much irradiation and/or smaller cells and/or more damage-susceptible cells (due to growth in minimal medium) could cause the growth stop. We observed that the bacterium is always attached somewhat underneath the bead, in this way catching more laser light than necessary (**Figure 8.2**). We attribute this to a different height of stable trapping of a bead and a bacterium. Improvements could be sticking the beads more on the ends of a bacterium (this would require traps created by both objectives), as well as using defined rich medium instead of minimal medium.

As mentioned above, a single tether could be made by chance to the region that will constrict. We managed to attach a DNA bead via a DNA tether to a dumbbell bacterium (data not shown). We found that rotation of the bacterium, as well as having to take into

account the positions of three beads (two polar handles and the DNA bead) makes the experiment difficult to analyze.

Without a specific mid-cell anchoring, other potential problems exist. If the OmpA β-barrel can freely diffuse in the OM, it will likely be pulled out of the "saddle point" formed by the constriction. Alternatively, if it is stuck to (existing) PG, such as full-length OmpA, then the invagination cannot be followed since this occurs through new synthesis of PG. Thus an OM protein that dynamically binds to the leading edge of the septum is required to keep the tether at the division site.

Additionally, an under-appreciated fact is that during division of a walled bacterium, the new poles are not shaped perfectly symmetric: often an angle between the long axes of the daughter cells is formed. This angle cannot be straightened out in the OT, and it is likely encoded in the PG cell wall. When attaching beads to such a bacterium, rotation becomes important and the actual invagination becomes more difficult to follow. For example, in an experiment where the "force probe" bead would be held at a constant position and a dividing bacterium must pull the bead out to constrict, the formation of an "septal" angle will (due to the freedom of rotation of the trapped beads) cause a shortening of the distance between bacterium and bead, and from the relaxation of the force in the construct it will appear as if the bacterium expands instead of constricts.

About the strength of the bead handles: as mentioned above, for a prolonged growing bacterium, IR exposure must be minimized. Thus, the polar handle beads must be very weak traps (e.g. 5 pN/μm). For a 1 μm diameter bacterium, assuming spherical caps, full division will typically displace both bead handles ~500 nm each. Thus, during division, their positions require continuous (manual) updating. The weak traps containing the handles limit the allowed force in the DNA tether. This prevents us from exerting forces > ~ 1 pN, and only the speed of constriction will likely be measured. A recent approach based on live cell imaging and careful image analysis was able to measure speed of constriction in *E. coli* up to a few hundred nm inwards, so this approach, if it would succeed, would not bring much news (Reshes et al. 2008).

Increasing the strength of the protein-DNA connection

This research is driven by interest in the effect of force on bacterial (cell-wall) growth and division. Assuming that indeed the SA-1 peptide-streptavidin bond can be described with the Bell model for molecular bonds (Chapter 7, Equation 1), with parameters thermal off-

rate koff= 10-3 s-1 and barrier width xb=0.5 nm, ~5 pN force is estimated to reduce the bond lifetime to 9 min (17 min at zero force).

Although forces in the order of a few pN are the forces expected for a force-generating Z-ring in the absence of an abundant motor protein, it is possible that higher forces are needed. It will definitely make the experiment easier, as after tether formation, division might not start immediately, whereas tether breakage at ~10 pN might occur within a minute. Thus, careful timing and control of the force (i.e. exceeding 10 pN will rapidly decrease the bond lifetime) are required for the SA-1 based interaction.

Genetically inserting the 15-amino acid biotin acceptor peptide (Beckett et al. 1999) into OmpA (as was previously done for LamB (Oddershede et al. 2002)) might be good way to increase the strength of the molecular construct. Although the *in vivo* biotinylation, which occurs only in the cytoplasm by endogenous biotin ligase (Chapman-Smith and Cronan 1999), might be too low to have sufficient biotinylated OmpA in the OM to efficiently form a DNA tether during a single molecule experiment, adding biotin and biotin ligase to the external medium can enhance this. That this can work has been shown recently (Howarth et al. 2005; Howarth and Ting 2008).

Effect of forces on a growing bacterium

If we extend the scope of our research question beyond the Z-ring and more general focus on the effect of force on the growth of the bacterial cell wall, then we no longer require a fusion protein that is localized at mid-cell, and different options come in sight.

For instance, directly attaching beads to growing bacterial filaments allows exertion of forces in the range of a few tens of pN at multiple points along the filament. Possibly, such forces are already sufficient to influence the shape of the bacterium as it grows. In preliminary experiments, we have developed a protocol in which biotin beads are attached specifically to a streptavidin-coated bacterial filament. Large diameter biotin beads are used to keep the laser focus sufficiently far away from the bacterium and to prevent growth inhibition. The resulting bead-coated filaments are shown in **Figure 8.3**.

If high forces exceeding a few hundred pN are required to influence cell wall growth, then a technique used to visualize forces during cellular adhesion to its substrate might be used. This technique makes use of surfaces of micro-fabricated transparent PDMS pillars (Tan et al. 2003). After calibration of the pillars, the deflection of such a pillar is a measure of the force exerted on it. In (Tan et al. 2003), the pillars (diameter 3 μm) had a spring

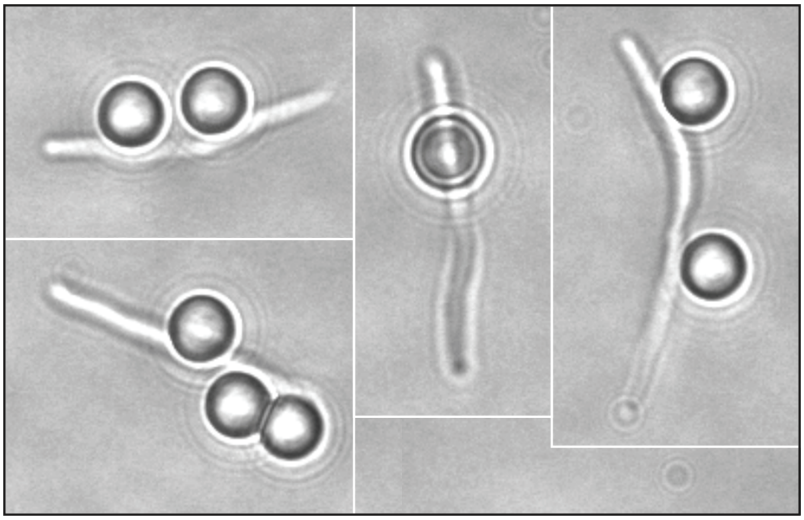


Figure 8.3. Attaching beads as handles to living filamented *E. coli*. Experimental protocol: LMC500 cells expressing OmpA-(SA-1) (on a pBAD plasmid) are grown overnight in Defined rich glycerol (DRy) medium with appropriate antibiotics. In the morning, cells are diluted 500x in fresh DRy. After ~1 hour, expression of OmpA-(SA-1) is induced through growth in the presence of 0.02% arabinose for 2 hours. Then filament formation is induced by addition of cephalexin (e.c. 10 μg/ml) and further growth for 2 hours. Subsequently, cells are labeled with streptavidin: Briefly, 1 ml of cells are concentrated to ~20 μl (~10^9 cells) and streptavidin is added (e.c. 50 μg/ml), incubated for 10 min at RT, washed 3 times and resuspended in ~20 μl. ~10^9 Biotin beads are also washed and resuspended in ~20 μl. 10 μl of cells is mixed with 10 μl of biotin beads (i.e. bead:cell ratio 1:1) and incubated for 10 min at RT. The mixture is diluted 100x and was imaged in brightfield in the optical tweezers setup.

constant of ~30 nN/μm, making the technique suitable for forces > ~1 nN. In (Roos et al. 2005) it is shown that such pillars can be coated with poly-L-lysine (PLL) to adhere microtubules. Thus, adhering bacteria on the PLL pillar heads and subsequently inducing filamentation (by blocking division with an antibiotic such as cephalexin) allows measuring the forces that can be generated by a growing bacterium, or alternatively, the forces required to affect shape by growing e.g. a curved filament.

Concluding remarks

We conclude that continuing the search for a mid-cell localized fusion protein in the OM is the direction that holds the greatest promise for the goal of measuring forces in a constricting *E. coli* bacterium. The creation of such a fusion construct will complete the list

of requirements that have to be met before forces can be measured. Using biotin-streptavidin recognition at the bacterial surface, and multiple dig-anti-dig interactions on the bead, a sufficiently strong DNA tethering can be made (**Chapter 7** and this chapter). Using the time-shared optical trapping assembly procedure presented in this chapter, the assembly of a viable dividing bacterium in between two trapped DNA-beads is expected to become a reality. The resulting force measurements will provide a unique way to characterize possible force-generation in a living cell.