REVIEW ARTICLE

Bacterial translocation motors investigated by single molecule techniques

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Abstract

Translocation of DNA and protein fibers through narrow constrictions is a ubiquitous and crucial activity of bacterial cells. Bacteria use specialized machines to support macromolecular movement. A very important step toward a mechanistic understanding of these translocation machines is the characterization of their physical properties at the single molecule level. Recently, four bacterial transport processes have been characterized by nanomanipulation at the single molecule level, DNA translocation by FtsK and SpoIIIE, DNA import during transformation, and the related process of a type IV pilus retraction. With all four processes, the translocation rates, processivity, and stalling forces were remarkably high as compared with single molecule experiments with other molecular motors. Although substrates of all four processes proceed along a preferential direction of translocation, directionality has been shown to be controlled by distinct mechanisms.

Introduction

Efficient translocation of DNA through narrow constrictions is important for bacterial genome maintenance/dynamics during processes as diverse as cell division, sporulation, and DNA transformation. Macromolecular transport is mediated by multimolecular translocases that include molecular motor proteins that convert chemical free energy into mechanical movement. A very important step toward a mechanistic understanding of these translocation machines is the characterization of their physical properties at the single molecule level including velocity, force generation, step size, processivity, dwell times, and directional switching. Furthermore, the effect of mutations in motor or accessory proteins on the physical properties can narrow down the molecular mechanism of motor proteins and their modes of interaction within a protein complex.

Four prominent single molecule techniques (laser tweezers, magnetic devices, atomic force microscopes, and single molecule fluorescence) have been developed to visualize and manipulate individual molecules (Lionnet *et al.*, 2006; Greenleaf *et al.*, 2007; Mickler *et al.*, 2008; Xie *et al.*, 2008). Single molecule fluorescence is highly useful for characterizing movement and gene expression in living cells but not for analyzing force generation (Xie *et al.*, 2008). Atomic force microscopies are usually applied to characterize processes that require high forces such as mechanical protein unfolding (Greenleaf *et al.*, 2007). Because the first two techniques have proven most useful for characterizing molecular motors, we will introduce these techniques in the first part of this review.

Several bacterial translocation motors have been addressed at the single molecule level, including FtsK, SpoIIIE, DNA import motors, and related type IV pili, VirE2, and SecB (Maier *et al.*, 2004a; Saleh *et al.*, 2004; Bechtluft *et al.*, 2007; Grange *et al.*, 2008; Ptacin *et al.*, 2008). In this review, we report recent advances that are relevant to the dynamics and molecular mechanism of the translocation machines and point out the role of single molecule experiments in deciphering their molecular mechanisms. We focus on four translocation machines that are relevant to the maintenance of the bacterial genome. FtsK and SpoIIIE are DNA translocation motors that pump DNA through the closing septum to ensure proper chromosome segregation during bacterial cell division and sporulation, respectively. Single molecule experiments *in vitro* revealed that both machines are remarkably

fast and powerful, and that they can control the direction of translocation by reading the chromosome polarity. Furthermore, we will discuss the DNA import machine that powers translocation of DNA during transformation and the related type IV pilus motor. These machines are relevant for generating the genetic diversity of bacteria. Single molecule experiments *in vivo* revealed that both machines are remarkably powerful, and we will discuss indications that the direction and speed are controlled by dynamic interaction between proteins within the multiprotein machines.

Single molecule techniques for characterization of molecular motors *in vitro* and *in vivo*

Mechanical force influences molecular movement

What information about molecular motors can we obtain from nanomanipulation experiments? The major function of a molecular motor protein is to generate movement and force. Conversely, when an external force is applied to a molecular motor, then the rate at which the mechanical steps of the motor cycle proceed can decrease or increase depending on the direction of the applied force. Application of high forces can potentially stop the motor and one can measure the stalling force of the motor using a suitable experimental setup. Further insight into the structure and mechanism of the motor may be gained by direct measurement of the elementary step length. Step sizes of molecular motors are typically in the range of several nanometers (10^{-9} m). Energy for translocation is usually provided by ion gradients, NTP hydrolysis, or binding energy. A simple estimation of the force generated by a molecular motor can be obtained by the ratio of this energy divided by the elementary step length of the motor. Assuming a step length of 1 nm and considering that the typical energy released by ATP hydrolysis in the cytoplasm is in the range of 80 pN nm (Nicholls & Ferguson, 2002), a typical value is $F = 8 \times 10^{-11}$N = 80 pN (about the weight of 1000 bacteria), provided that the motor works at 100% efficiency. The efficiency of the motor is defined by the ratio of mechanical work (i.e. stalling force multiplied by the step length) and the chemical energy. Note that this value can be very high (up to almost 100% for F1 ATPase). Specific tools have been developed to apply and measure such low forces, and to detect steps at the nanometer scale. Two of them are briefly described in the following.

Laser tweezers

Laser tweezers (also optical tweezers, optical traps) are usually built around light microscopes. In laser tweezer setups, the objective is used to focus a laser beam to a diffraction-limited spot in the image plane. Dielectric, transparent, objects (such as silica or latex beads or bacteria) experience an optical force that is usually separated into two components: the scattering force pushes the object into the direction of light propagation and the gradient force pulls the object into the direction of increasing light intensity (Sheetz, 1998). If the geometry of the experimental setup is chosen carefully, the gradient force dominates over the scattering force and dielectric objects can be stably trapped near the focus of the laser light. This physical property has been used very successfully to trap objects between 5 nm and several micrometers in diameter (Neuman & Block, 2004). What is the physical basis of the optical force? Depending on the size of the objects, there are two intuitive approaches to understanding force generation. If the object is much smaller than the wavelength of the laser light (which is typically in the near-infrared region *c.* 1 μm), it develops an electric dipole in response to the electric field and is drawn up intensity gradients in the electric field toward the focus. If the object is much larger than the wavelength, one can consider them as lenses, refracting the rays of light and redirecting the momentum of the photons. The resulting recoil draws them toward higher flux of photons near the focus. This recoil is imperceptible for macroscopic lenses, but can have a huge influence on objects with a size of micrometers or lower.

This relatively simple principle can be applied to manipulate micrometer-sized objects, for example, for cell sorting or to apply calibrated forces to biological cells (Sheetz, 1998). Laser tweezers have been advanced to generating multiple traps (Grier, 2003) and traps that can rotate particles (Nieminen *et al.*, 2007).

Most importantly, for this review, laser tweezers have been applied very successfully to study the rates, force generation, processivity, basic step size, and mechanochemical coupling of molecular motors. To obtain a high spatial resolution, the tracking methods for detection of the position of the bead (Neuman & Block, 2004) and the stability of the setup have been continuously improved in recent years. For high-resolution tracking and simultaneous force measurement, micrometer-sized beads are usually used as handles (Fig. 1a and b). They are attached to the molecular motor either directly as in the case of kinesin or myosin (Howard, 2001) or through tethers that are often made up of DNA or protein filaments such as titin (Sotomayor & Schulten, 2007) or pili (Maier, 2005). Several alternative methods have been developed for fast position detection of beads (Neuman & Block, 2004; Di Leonardo *et al.*, 2007). Most methods rely on fast photodiodes and allow position detection at rates exceeding 10 kHz. The position of the bead with respect to the center of the laser tweezers as a function of time can then be used to extract information about the

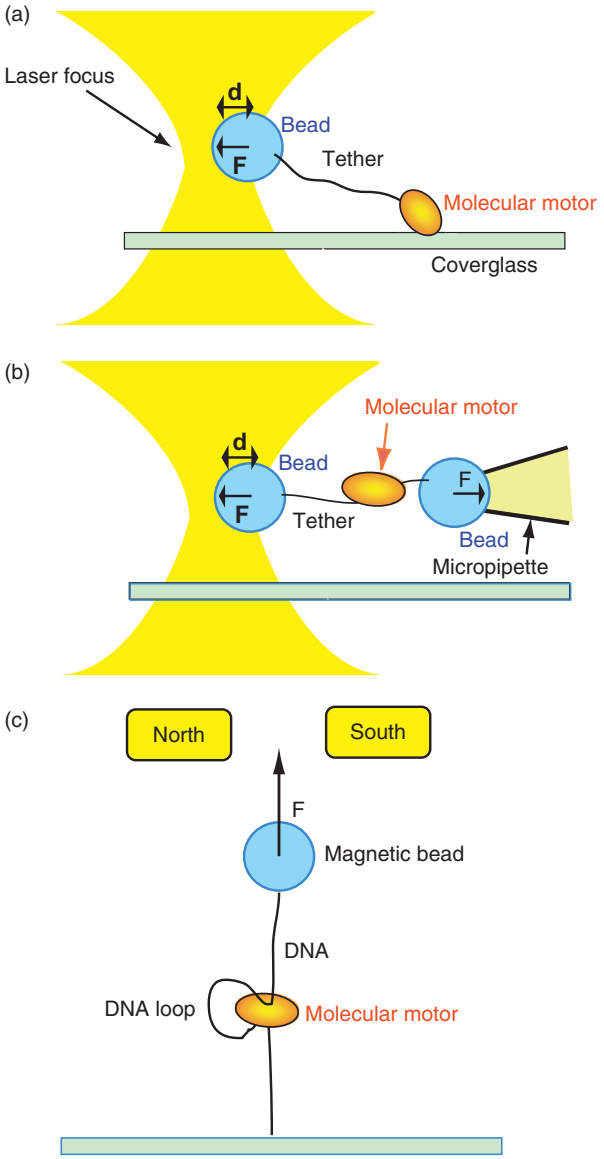

Fig. 1. Setups typically used for characterization of molecular motors. (a) Basic laser tweezers. The molecular motor is attached to a microscope coverglass and tethered to a micrometer-sized bead via DNA or protein. As the motor pulls on the tether, the bead is displaced from the center of the laser trap. The absolute value *d* is proportional to the optical restoring force and can be measured with high accuracy. (b) Laser tweezers setup used for characterization of FtsK. The motor is attached to a bead held in a micropipette. (c) Setup of magnetic device. A DNA molecule is attached to a glass substrate and pulled, thanks to the magnetic bead attached at the other end. The sample is placed below permanent magnets that produce the field necessary to pull on the bead. Rotation of the magnets produces supercoils in the molecule.

velocity and directionality of molecular motors. If the signal to noise ratio is high enough, individual steps in the motor cycle can be resolved, yielding a direct measure of step size (Moffitt *et al.*, 2008) and dwell time distributions, which can provide important information about the coordination of different subunits within a molecular motor complex (Asbury *et al.*, 2003).

Within several hundreds of nanometers around the center of a laser trap, the optical restoring force increases linearly with the displacement of the bead from the center of the laser trap. Thus, the determination of bead position with respect to the center of the trap also yields the force applied on the molecular motor. While a molecular motor pulls the bead away from the center of the laser trap, the optical restoring force increases as a function of time. To keep the force constant, different methods for force clamping have been developed (Lang *et al.*, 2002; Greenleaf *et al.*, 2005).

Without sophisticated stabilization of the setup, the spatial resolution is often limited to several nanometers due to microscope drift and various sources of environmental noise. Thus, steps in the motor cycle can only be resolved for motors with large step sizes in the range of several nanometers including kinesin or myosin (Moffitt *et al.*, 2008). The setup may be stabilized through monitoring the surface of the microscopy chamber at 0.1 nm resolution at 100 Hz (Carter *et al.*, 2007). Currently, the most efficient method to decouple the laser trap from environmental fluctuations is to use two optical traps yielding a resolution of 0.1 nm, provided that the sampling time is long enough.

Magnetic devices

As one can experience with magnets on refrigerators, a magnetic material, such as iron, is subjected to a force in the presence of magnetic fields. At the same time, a compass indicates the north pole, as a result of the torque produced by the action of the earth's magnetic field.

These simple facts have been used to apply a force and induce supercoiling on a single DNA molecule (Strick *et al.*, 1996). With this technique, named ambiguously magnetic tweezers (they are not real tweezers with a 3D control of the position of the trapped object), the magnetic field is produced by permanent magnets and the magnetic material is embedded in a small bead coated with proteins. This bioactive outer shell is used to attach one end of a DNA molecule while its other extremity is bound to a functionalized glass surface (Fig. 1c).

The magnetic force on the bead is then transmitted to the DNA molecule, which is subsequently extended. Rotation of the magnets, thanks to computer-controlled motors, produces a synchronous rotation of the bead. This provides a very simple way to induce controllable and reversible supercoiling of the nucleic acid. Experimentally, the force acting on the superparamagnetic bead increases with decreasing distance between the magnets and the bead. By varying the distance between the magnets and the beads, one can apply forces from fractions of piconewton to hundreds of

piconewtons (Strick *et al.*, 1998). The number of supercoils added by rotation of the magnets has no technical limit: supercoils can be added as long as DNA does not break. This fact has been used to study structural transitions of DNA (Allemand *et al.*, 1998; Strick *et al.*, 1999).

Magnetic tweezers have the advantage of applying a constant force on the DNA during molecular motor action without any feedback. Results are usually simpler to interpret in a constant force system. Moreover, because the magnetic field extends over a relatively large region, this force is applied to many molecules at the same time, which allows, in principle, more data output (Ribeck & Saleh, 2008). A drawback is that measurements are performed through a video camera and are therefore much slower than optical tweezers (Neuman *et al.*, 2005).

In actual experiments, DNA molecules are typically a few kilo base pairs and the bead size is about 1 μm. The beads are observed under a microscope and the position of the bead is monitored in three dimensions and in real time. This allows determining the force acting on the molecule and more interestingly for molecular motors, the extension of the molecule as a function of time. For molecular motors that modulate the extension of DNA, reduction of the extension as a function of the time will simply provide motor speed (Lionnet *et al.*, 2006).

Limitations of single molecule manipulation tools using beads as handles

The resolution of single molecule experiments that use beads as probes for detection of molecular movement is not exclusively determined by the speed of the detector because the small bead is subject to Brownian motion. The signal to noise ratio depends on various experimental parameters, including the stiffness of the tether, the size of the bead, and the speed of the detector. Therefore, to obtain a certain signal to noise ratio for positional detection, one has to perform temporal averaging, and thus the spatial resolution can only be increased at the cost of temporal resolution (Moffitt *et al.*, 2008). For example, to obtain the basic step size of RNA polymerase of 0.37 nm, temporal averaging over *c.* 1 s was necessary (Abbondanzieri *et al.*, 2005).

Another problem specific to laser tweezers is potential photo damage of the biological material. Because there is a window of relative transparency in the near-infrared region, infrared lasers are usually chosen for the analysis of biological material (Neuman & Block, 2004). Nevertheless, the forces that are accessible through laser tweezers are limited by the power of the laser that can be used without damaging the biological samples.

Laser tweezers and magnetic devices have been very successfully used to probe and manipulate molecular motors *in vitro*. However, in living cells, motors are usually part of complex machines and physical properties are likely to be influenced through the interaction between multiple proteins. The trick of using small beads as handles to probe molecular motors *in vivo* is so far only applicable for molecular motors that reside near the cell membrane and have a polymeric handle at the external side of the cell, such as DNA, flagella (Block *et al.*, 1989), or pili (Maier, 2005).

FtsK and SpoIIIE

Functions of FtsK and SpoIIIE

FtsK and SpoIIIE are molecular motors involved in DNA transport inside bacteria. FtsK acts during symmetric cell division (Begg *et al.*, 1995; Bigot *et al.*, 2007), whereas SpoIIIE is essential during asymmetric division (sporulation) (Wu & Errington, 1994, 1997; Sharp & Pogliano, 1999, 2002; Errington *et al.*, 2001). In both cases, the motors are located at the septum (asymmetrically in the case of SpoIIIE), the location where the cell membrane pinches before cell separation (Fig. 2a and b). As bacterial chromosome segregation (Sherratt, 2003) is not as well coordinated with DNA replication and membrane separation as in eukaryotes, some DNA can be, and indeed is in the case of sporulation, trapped when the septum closes. FtsK and SpoIIIE are thought to use the energy of ATP hydrolysis to pump the potentially trapped DNA into the right cell before cell separation. Please note that they do not have to provide all the work required for migrating DNA to their new positions, because the chromosome is already partially in the correct cell, but this proportion can nevertheless be quite high in the case of SpoIIIE. Furthermore, homologous recombination events during replication can induce chromosome dimers (Sherratt, 2003). In the case of *Escherichia coli*, this means that instead of having two circular chromosomes of about 4 Mbp (4.6 Mbp for *E coli* K12), replication produces only one circular chromosome with double length (Fig. 2a). This configuration will block cell division because an effective septum closure will result in DNA chromosome breakage. An important function of FtsK is to catalyze the biochemical reaction that resolves these dimers through a physical contact with the two site-specific recombinases XerC and *D.* In *Bacillus subtilis*, CodV and RipX are the recombinases involved, but in this case a direct evidence for the implication of SpoIIIE is still missing (Errington *et al.*, 2001; Sciochetti & Piggot, 2000). Similarly, one must notice that FtsK mutants defective in ATPase activity have a death rate similar to, and not higher than, natural chromosome dimer formation and thus a direct role of FtsK apart from dimer resolution still awaits experimental proof despite its strong sequence similarity to SpoIIIE, for which the role in DNA pumping has been clearly demonstrated.

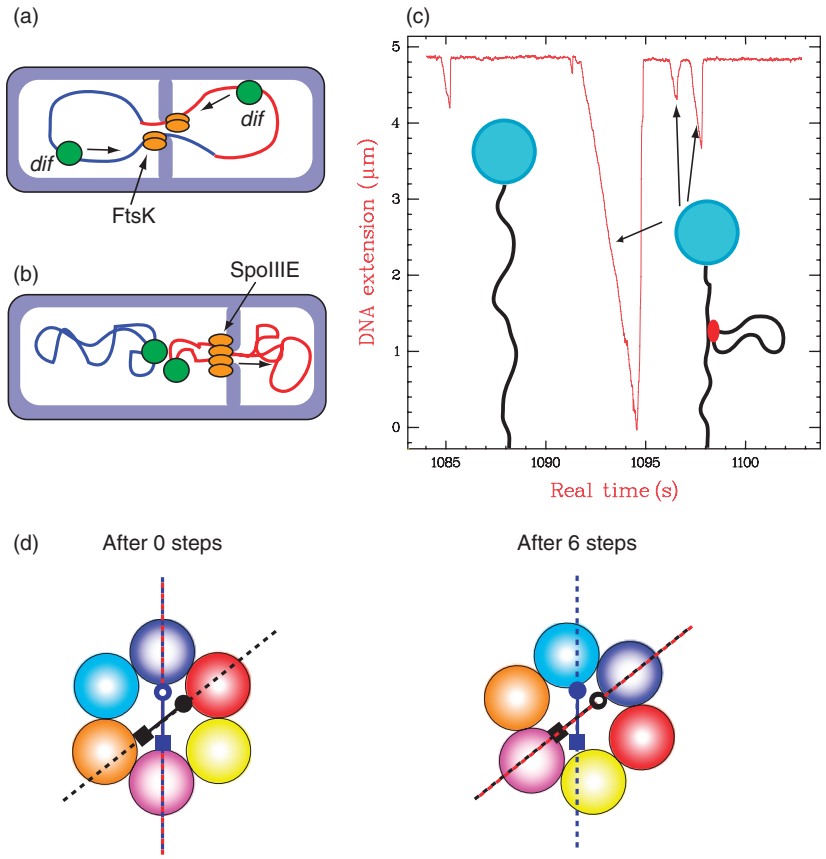

Fig. 2. FtsK and SpoIIIE. (a) Schematic view of the role played by FtsK during chromosome dimer resolution. FtsK localized at the septum pumps DNA in order to bring two specific sites named *dif* in proximity. It will then catalyze the reaction of recombinases at the *dif* sites (not represented), leading to dimer resolution. (b) Whereas FtsK acts during a symmetric cell division, SpoIIIE pumps DNA during sporulation in a very asymmetric fashion. It pumps *c.* 70% of *Bacillus subtilis* chromosome into the forespore. (c) Typical event of FtsK translocation on a DNA molecule with the magnetic device. FtsK (red spot) binds to DNA at two points and extrudes a loop. As a result, the end-to-end distance of the molecule decreases fast because of motor activity. At some point, depending on the processivity, one of the attachment points breaks and the molecule reverts to its initial extension. (d) Model for DNA supercoil induction proposed by Massey *et al.* (2006). FtsK forms hexamers. Its monomers are then arranged at 60° angles against each other (large circles). The FtsK step size is *c.* 2 bp. After six steps the motor is then positioned 12 bp further on the DNA. But as DNA helical pitch is 10.5 bp, the motor has to perform a rotation equivalent to 12–10.5 = 1.5 bp (i.e. *c.* 51°) in order to bind to a similar site on the DNA. At step 0 (left) the blue monomer is in contact (white spot) with a base pair represented in blue. The next base pair where the same monomer (blue) will be in contact after six steps of 2 bp is represented in black. The angle with respect to the initial base is about 51°. After six steps (right), the hexamer has to rotate by this angle in order to contact the black base in the same manner. This corresponds to an average rotation of 4.25° bp^{-1} translocated.

Studies concerning FtsK showed that structurally these motors can be thought of as three parts. The N-terminal hydrophobic part anchors the motor in the cell membrane and locates it at the septum. A less-conserved linker connects the N-terminal part and the C-terminal cytosplasmic ATPase region. This region forms hexameric rings (Aussel *et al.*, 2002; Massey *et al.*, 2006) and translocates along DNA. It is composed of three different domains: α, β, and γ. The first two function as the motor using ATP hydrolysis to produce movement (Massey *et al.*, 2006), whereas the latter interacts with DNA sequence (see the end of this section).

Dynamics of FtsK and SpoIIIE

Bath *et al.* (2000) reported the first direct experimental proof that SpoIIIE translocated DNA using a truncated version of this motor retaining mainly the C-terminal part. Their conclusions were based on the observation that in the presence of ATP, the action of the motor on a circular DNA in the presence of *E. coli* topoisomerase I (TopoI) created positive supercoils. Considering the fact that SpoIIIE has no topoisomerase activity, two explanations could be proposed for this effect: either there is cooperative binding of the proteins with a binding, generating DNA torsion, or the

motor binds to two sites on the DNA and creates both positive and negative supercoils during its translocation. In this second hypothesis during the translocation, the (algebraic) sign of supercoiling depends on the position on the DNA: one sign on the already translocated DNA, and the opposite on the other part. As TopoI relaxes negative supercoils, positive supercoils can be accumulated during translocation. As no cooperative binding has been observed, the latter hypothesis is then the correct one. A more direct and more intuitive demonstration of FtsK translocation was cruciform extrusion (Aussel *et al.*, 2002) or triple helix displacement (Levy *et al.*, 2005).

One of the advantages of single molecule techniques is that they readily provide a dynamic view of protein action and a more natural way of detecting the heterogeneity among the proteins. For example, the value of the speed in bulk experiments is obtained from a large number of molecules and is thus an average on all individual molecules although they may behave differently. In bulk, heterogeneity and subpopulations can also be observed, but less naturally. Even if individual motors would behave similarly, temporal variation of the motor speed along a displacement, due to a sequence effect for example, would be much more tedious to measure (see e.g. some studies on the pausing of RNA polymerases: Davenport *et al.*, 2000; Neuman *et al.*, 2003; Herbert *et al.*, 2006).

In the specific case of FtsK or SpoIIIE, bulk experiments cannot quantify various important parameters of motor translocation. For example, a very good bulk test for protein translocation is triplex displacement, where a translocase strips a triplex from DNA. In the case of FtsK, however, it cannot be used to measure the speed of the translocase because one cannot preload FtsK (or SpoIIIE) at a precise distance, i.e on a precise sequence, from the triplex. Similar arguments can be used for the processivity. Therefore, single molecule techniques, as described above, can provide a better quantitative view on the dynamical aspects of the motor's action. Even if bulk experiments provided evidence for translocation and supercoil production by SpoIIIE or FtsK, they cannot provide any quantitative information concerning the speed or the processivity, nor can they provide quantitative information about the link between translation and rotation around DNA as implied by induction of supercoiling (this requires the exact knowledge of the distance travelled by the motors). Of course, to be meaningful, single molecule data must be obtained from a significant number of molecules providing statistically similar results.

As shown in bulk experiments, through the previously mentioned production of positive supercoils on plasmids in the presence of TopoI, the motor (or more precisely its *in vitro* truncated version) binds to two sites on the DNA, forming a loop and then translocating along the DNA, increasing the loop size. Using optical or magnetic tweezers, linear DNA can be stretched between two beads that are attached to both extremities of DNA (Fig. 1). As explained in the previous section, these setups can be used to measure the end-to-end distance of the DNA molecule as a function of time, while a molecular motor translocates DNA. When this DNA molecule is stretched, the part of the molecule between the two binding sites of FtsK or SpoIIIE will not be subjected to force and will not contribute to the end-to-end extension of the molecule. Translocation of the motor will simply increase the proportion of the molecule between the binding sites, and as a result, the end-to-end extension of the DNA will decrease (Saleh *et al.*, 2004). To be sure that a single motor is active at a time one has to set the FtsK concentration so that activity events are well separated (compared with the event duration), ensuring that the probability that two motors are active is very low (Saleh *et al.*, 2004; Seidel *et al.*, 2004). Under some conditions (Pease *et al.*, 2005), large FtsK aggregates of molecules (blobs) formed, making the complex visible and ensuring the detection of motor localization on the DNA. Even if many molecules were present in these blobs, their speeds were similar to the 'real' single molecule conditions, meaning that despite the large number of proteins in the blob, only one was active in the blob. Nevertheless, for a more precise and more direct localization of the motor on the DNA molecule, experiments with fluorescently tagged motors are still required.

We must add that DNA loops formed by FtsK in the *in vitro* experiments, where a truncated version of the protein is used, are a consequence of head-to-head linking of two FtsK hexamers. Head-to-head linking has been observed in EM (Massey *et al.*, 2006), but has, at least to our present knowledge, not been observed *in vivo*, where the role of loops would be currently unclear.

Single molecule techniques allow monitoring the extension of DNA as a function of time to access motor speed as a function of force or other biochemical conditions. A typical signal is presented in Fig. 2c. The first element revealed by these techniques was that these motors are extraordinarily fast, about $2\,\mu m\,s^{-1}$ or about $7\,Kbp\,s^{-1}$ (Saleh *et al.*, 2004; Pease *et al.*, 2005; Ptacin *et al.*, 2008)! The speed strongly exceeds the speeds reported to date for any other DNA translocase (Wang *et al.*, 1998; Spies *et al.*, 2003). The maximum change in DNA extension for each event represents the individual processivity. The value averaged over multiple events yields the average processivity usually obtained in bulk experiments (Saleh *et al.*, 2004). The general advantage of this method is that it allows the observation of the variability between different motors that allows in principle to detect heterogeneity in the population. In this particular case, there is another obvious advantage: after any burst of activity the system goes back to the initial point, i.e. the DNA loop between the two binding sites of the motor enzymes disappears (Saleh *et al.*, 2004; Pease *et al.*, 2005).

This means that without monitoring the extension in real time like single molecule techniques do, one may underestimate the speed and processivity, and in the worst cases, totally miss the activity.

Force generation of FtsK and SpoIIIE

Another surprising property of these translocases is that the speed does not decrease with forces as high as 40 pN (Pease *et al.*, 2005). This means that the rate-limiting step of the motor does not involve movement up to these forces. Otherwise, the force opposed to the movement would increase the energy required for the movement and thus reduce the kinetics of the movement. Stalling of the motor was not observed even at forces above 60 pN, but the stalling force could not be measured because at these forces the motor unbinds before stalling (Pease *et al.*, 2005). This value is to be compared with the 5 pN required to stall myosin II on actin or 8 pN to stall kinesin on microtubules (Svoboda & Block, 1994; Howard, 2001). A potential role for this impressive value can be the need for moving a large part of a chromosome against a strong friction opposing the movement. Furthermore, it has been demonstrated that SpoIIIE is able to remove an RNA polymerase from DNA (Marquis *et al.*, 2008), and thus high force generation can be used to remove obstacles on the DNA.

Supercoiling activity of FtsK

As we already mentioned, at least *in vitro* these motors bind to two distinct sites on the DNA, and as the motor works, it generates a given supercoiling in front of it and the opposite within the DNA loop between the two sites' motor attachment. Single molecule techniques offer the possibility to generate supercoiling very easily. This property has been used to quantify the rotation of *E. coli* FtsK around the DNA as it translocates along the molecule (Saleh *et al.*, 2005). How can rotation be measured in a purely mechanical approach? Of course, supercoiling generates supercoils! When they appear on an extended DNA molecule, these supercoils reduce DNA extension (there is a small threshold in rotation for this reduction that we neglect here for clarity). The value of this reduction depends slightly on the pulling force, and salt conditions and has an average value of *c.* 40 nm per turn (Charvin *et al.*, 2004). Hence, if using magnetic tweezers we impose N supercoils on a DNA molecule, its end-to-end extension is reduced by $N \times 40$ nm. Let us now assume (as it will be the case in the experiments) that FtsK works on this molecule and generates positive supercoils (outside the loop) while translocating. Let us denote D as the distance over which FtsK translocates in order to generate a single positive supercoil. When FtsK has moved over a distance D on the molecule, the DNA extension has been modified for two reasons. First, as the size of the loop between the binding points of the motor has increased by a distance D, the molecule has shortened by an amount D (to be corrected with a function depending on the pulling force), as used previously to measure FtsK speed. However, at the same time, as one positive supercoil has now been added to the N negative supercoils there are now only $N-1$ supercoils on the molecule and thus its extension has increased by 40 nm. Thus, DNA extension has been modified by an amount of $+40-D$. If D is smaller than 40 nm, and it is indeed the case in the experiments, then the expansion of the DNA loop results in an increase of DNA extension. Quantitative analysis of the experimental signal showed that the motor indeed produces positive supercoils as it translocates with an amount equal to one supercoil induced for 150 bases translocated. For comparison, a groove-tracking protein would produce one supercoil for 10.5 bases (Seidel *et al.*, 2004). This value is quite surprising because it means that creation of supercoils is important as it has a very well-defined value, but at the same time it does not correspond to a motor following the helical pitch as one may expect for a sequence reading motor.

A potential explanation is as follows: FtsK activity inside the bacterium should in principle produce supercoils that could perturb chromosomal supercoiling density. Intriguingly, it has been reported that the measured value for FtsK is exactly opposite to the supercoiling naturally present in *E. coli*. For this specific value, when the motor translocates along a given distance, the supercoils produced by this movement compensate (they cancel) exactly the natural supercoiling in this portion of DNA. Thus, the initially present supercoils (or more exactly the difference between these ones and the one generated by FtsK) do not have to be compacted in the still to be translocated DNA and thus the supercoiling density (number of supercoils per length) is not modified in this part of the DNA. The measured value for the coupling rotation/translation is then the only value where the supercoils produced have surprisingly no effect on the chromosomal supercoiling density. This conclusion implies that the motor's structure evolved to generate the specific value of one supercoil per 150 bases. This value is in agreement, for the sign of the supercoiling induced, with the prediction of the rotary inchworm translocation model proposed by Massey *et al.* (2006) (Fig. 2d). However, with a motor's step size of 2 bp deduced from the structure, this model predicts the generation of only one supercoil for 84 bases translocated. This evolutionary argument is still to be confirmed by similar measurements with other bacterial species than *E. coli*.

Control of directional movement by FtsK and SpoIIIE

Considering that FtsK and SpoIIIE move along double-stranded DNA, they can move in any direction on the DNA

because the molecule has no directionality. Indeed, at a low force on a DNA sequence containing no specific sequence FtsK (at least its *in vitro* version) can even change its direction during its activity and so it can move in both directions along the same sequence (Saleh *et al.*, 2004; Pease *et al.*, 2005). However, considering the role of these motors inside the bacteria there must be a preferential direction of the movement. In the case of SpoIIIE, it could be argued that with the division being asymmetric, the localization of the protein could be asymmetric to impose a given direction to the movement. However, the argument cannot hold for FtsK family proteins where the division is symmetric. In fact, recent experiments demonstrated directly that the DNA sequence orients the motor (Bigot *et al.*, 2005; Levy *et al.*, 2005; Pease *et al.*, 2005; Ptacin *et al.*, 2008) and that the sequence is read by the γ-subunit of the motor (Ptacin *et al.*, 2006; Sivanathan *et al.*, 2006). It has already been shown that inversion of long sequences along the chromosome in *E. coli* could affect FtsK activity on dimer resolution (Corre & Louarn, 2002). It took quite a while to isolate the sequence involved (Bigot *et al.*, 2005). When used in single molecule experiments, these sequences (Bigot *et al.*, 2005; Levy *et al.*, 2005; Pease *et al.*, 2005; Ptacin *et al.*, 2008) induce the confinement of the motor between a physical barrier, such as a solid surface, another bound FtsK (Lowe *et al.*, 2008) and the specific sequence, or between two sequences oriented in opposite directions. In other words, in these experiments, the processivity is highly affected by these sequences. They have been named KOPS (FtsK Orienting Polar Sequence) or FRS (FtsK recognition sequence) in the case of FtsK and SRS in the case of SpoIIIE. How can these sequences orient the motor? Two possibilities have been proposed. The first one relies on the preferential oriented binding of the motor, and the second one implies that the motors can bind anywhere on the DNA and translocates until it encounters the KOPS or a similar sequence and then, after a conformational change, moves in the direction given by the sequence. The second case could indeed be true, arguing simply from single molecule experiments, because no specific sequence is required for observing motor activity. In our opinion, a decisive argument in favor of the first interpretation came from the observation in bulk experiments, that activity increased in the presence of KOPS compared with the activity of a similar DNA without KOPS (Bigot *et al.*, 2006). This argument is reinforced by the structure of the protein bound to the KOPS sequences that seems to preclude the recognition of the sequence when encountered in the wrong orientation and by other more complex biochemical experiments (Lowe *et al.*, 2008). An important aspect of sequence reading is that sequence recognition is not 100% efficient (Saleh *et al.*, 2004; Bigot *et al.*, 2005; Levy *et al.*, 2005; Ptacin *et al.*, 2008), meaning that to observe a very strong effect three consecutive sequences were required in the experiments. Otherwise, their presence could even be missed (Saleh *et al.*, 2004).

Type IV pilus dynamics

Functions of type IV pili

In most naturally transformable bacterial species, type IV pilus proteins are required for transformation as described in the following chapter. Because it is currently unclear whether extended pili participate in DNA transformation or whether they form part of a structurally distinct DNA import complex, we discuss type IV pili separately from DNA transformation. Apart from supporting transformation, type IV pili are among the most widespread bacterial colonization factors and they support virulence in many human pathogens. They mediate attachment to host cell surfaces and abiotic surfaces, power surface motility, and support biofilm formation.

Structure of type IV pili

Type IV pili are polymers with a length of several micrometers and an outer diameter of *c.* 5–8 nm (Craig *et al.*, 2004) (Fig. 3a). Their major compound is the *P*-shaped pilin monomer, which consists of a very hydrophobic tail and a head that is partially exposed to the environment (Parge *et al.*, 1995). This general architecture is shared by pilins from many different organisms, although the surface properties of the pilus vary (Craig & Li, 2008). A combination of X-ray crystallography, electron microscopy, and computational modelling suggests that the hydrophobic subunits are twisted in a helical array in the core of the pilus filament and thus anchor the head domains, which form the outer surface (Craig *et al.*, 2006). The effective length of one pilin subunit in the pilus is *c.* 1 nm. Simulations suggest that the outer surface of the pilus filament exhibits grooves with positive charges that could support binding of negatively charged DNA (Keizer *et al.*, 2001; Craig *et al.*, 2006), thus facilitating DNA binding during transformation. Please note that the efficiency of direct binding of DNA to pili is currently disputed (van Schaik *et al.*, 2005; Assalkhou *et al.*, 2007), and the role of direct binding of DNA to pili remains unknown.

Molecular motors supporting assembly and disassembly of the pilus

The length of a type IV pilus is dynamic and dynamics is controlled by a complex machine consisting of *c.* 15–20 proteins. To our current knowledge, six proteins are required for pilus assembly, one protein is essential for pilus disassembly (PilT), at least eight proteins influence the probability of pilus disassembly and/or maturation of the pilus, and one protein is necessary for export of the pilus from the periplasm (Wolfgang *et al.*, 1998, 2000; Morand

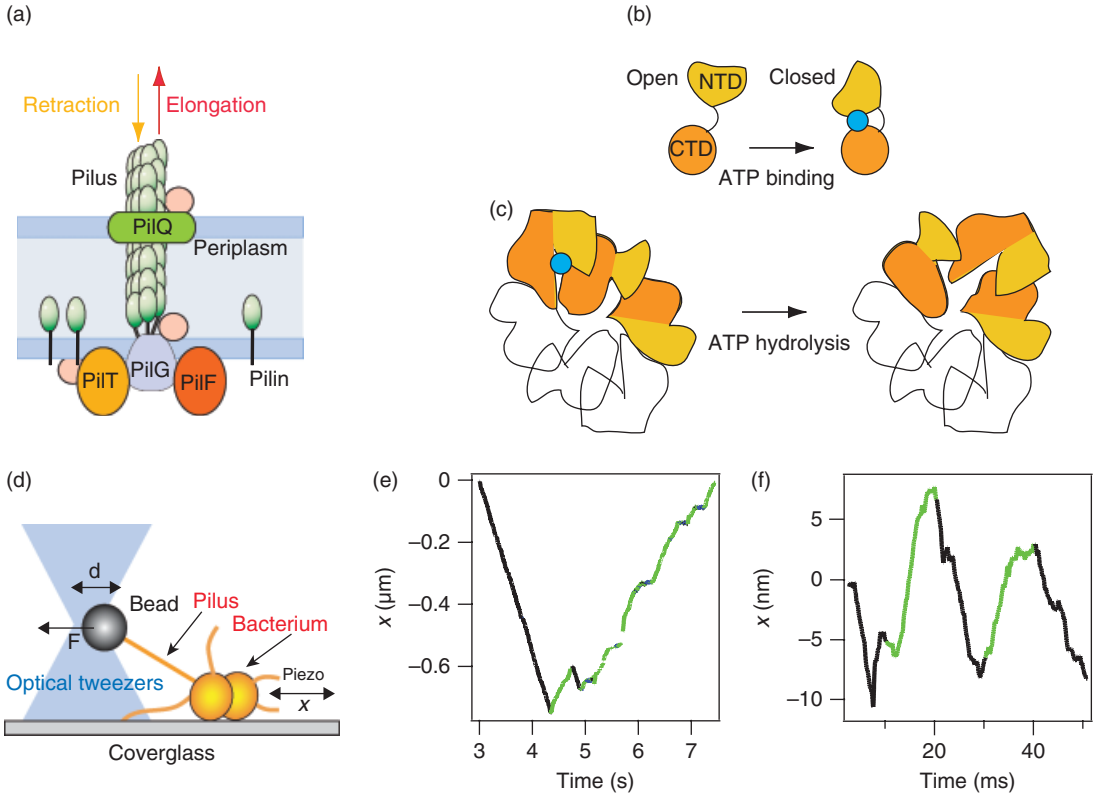

Fig. 3. Type IV pilus dynamics. (a) Basic elements of the type IV pilus machine. Pink disks denote proteins that modulate the probability of pilus retraction. Please note that the full type IV pilus system consists of at least 15–20 proteins. (b) Sketch of putative domain movement in a PilT monomer upon ATP binding. CTD, C-terminal domain; NTD, N-terminal domain; blue disk, ATP. (c) Sketch of putative deformation of the PilT hexamer upon ATP hydrolysis (adopted from Satyshur *et al.*, 2007). (d) Sketch of a single molecule assay for studying type IV pilus dynamics. *Neisseria gonorrhoeae* binds to a glass surface. A bead in a laser trap is approached to the bacterium and pilus retraction displaces the bead from the center of the laser trap. An active force clamp can be introduced by keeping the distance *d* constant while moving the bacterium with a piezo stage by a distance *x* while the pilus changes its length. (e) and (f) Typical examples of pilus length change *x* with force clamp as a function of time at external forces of 100 pN. Black lines, retraction; green lines, elongation; blue lines, pausing (e) Switching between retraction and elongation occurred at a time scale of seconds. (f) Switching between retraction and elongation occurred at a time scale of milliseconds.

et al., 2004; Carbonnelle *et al.*, 2005, 2006; Winther-Larsen *et al.*, 2005). Because the proteins and their function in pilus assembly have been discussed in detail in a recent review (Pelicic, 2008), we concentrate on the putative molecular motors in this review. Please note that homologous proteins have different notations in different bacterial species and throughout this review we will adopt the nomenclature for *Neisseria gonorrhoeae* (gonococci).

Pilus assembly is supported by an ATPase called PilF in *N. gonorrhoeae* (Freitag *et al.*, 1995). The antagonist of PilF is called PilT, and is required for retraction of pili into the cell body (Wolfgang *et al.*, 1998) (Fig. 3a). Both proteins belong to the family of typeII/typeIV hexameric secretion ATPases, including a Walker A and a loosely defined Walker B box. Although disassembly during retraction has not been demonstrated directly, it is highly unlikely that the fiber extends into the cell body due to its length of several micrometers and its persistence length of about 5 μm (Skerker & Berg, 2001). *In vitro*, NTPase activity has been shown for PilT (Herdendorf *et al.*, 2002) (Jakovljevic *et al.*, 2008). In *N. gonorrhoeae* and *Aquifex aeolicus*, PilT formed hexameric rings *in vitro* with an outer diameter of 10.5 nm and an inner diameter of 1.5–3.5 nm (Forest *et al.*, 2004) and formation of oligomers was highly cooperative with *A. aeolicus* (Herdendorf *et al.*, 2002). The question of whether the motor hexamers can bind multiple ATPs was addressed in enteropathogenic *E. coli* (Crowther *et al.*, 2005). The hexameric PilF ortholog in EPEC has been shown to bind six molecules of ATP *in vitro*.

Recently solved crystal structures of *A. aeolicus* PilT provide a novel insight into the potential mechanism for chemomechanical energy transduction (Satyshur *et al.*, 2007). The subunits showed bilobed structures connected by a hinge region (Fig. 3b). Different structures were solved and with all structures the subunits arranged into hexameric

rings (Fig. 3c). However, only the asymmetric structure possessed an active subunit conformation, which is likely to support ATP hydrolysis. In this conformation, two of the subunits are in an open conformation in which the N-terminal lobe is twisted by *c.* 70° from the C-terminal lobe about the hinge, resulting in a 1.5 nm shift of the N-terminal domain with respect to the C-terminal domain. It is therefore tempting to speculate that the hinge acts as a lever arm that amplifies small motions during ATP hydrolysis into nanometer-range movement of PilT that could then be transmitted to the moving pilus. This hypothesis is further supported by the fact that the length of a pilin subunit in the pilus is *c.* 1 nm. Movement may be transmitted through a membrane protein from the cytoplasmic PilT (Assalkhou *et al.*, 2007) to the periplasm (Collins *et al.*, 2007; Craig & Li, 2008).

Force generation by type IV pili

Dynamics of type IV pilus length change has been characterized indirectly by observing twitching motility, sensibility to phages, or adhesion to mammalian cells. However, to characterize important physical parameters of pilus dynamics including velocity, force generation, or control of direction, pilus dynamics must be addressed at the level of individual pili. To this end, Merz *et al.* (2000) developed an assay based on laser tweezers that allowed following pilus retraction in real time. The idea was to attach a bacterium (*N. gonorrhoeae*) to the surface of a microscopy cover slide and then approach a micron-sized latex bead to the bacterium (Fig. 3d). Once pili bound to the bead and retracted, the bead was displaced from the center of the laser tweezers and by following the position of the bead as a function of time they were able to measure the shortening of the pilus as a function of time. They found that the retraction velocity was in the range of *c.* 1 $\mu m\, s^{-1}$ and that pilus retraction generated a mechanical force. They also demonstrated that the retraction protein PilT was necessary for pilus retraction.

One important question was how many pili retracted simultaneously to generate a high mechanical force and how much force one individual pilus can generate by retraction. To address this question, laser tweezers were used to investigate force generation in an *N. gonorrhoeae* strain where the pilin gene promoter was derepressible (Maier *et al.*, 2002). With this strain, the stalling force at different levels of pili per cell was independent of the pilus concentration. The histogram of stalling forces was monomodal even for the wild type and independent of the number of pili per cell. This experiment showed that even in the wild-type, retraction was generated by individual pili that could generate forces in the range of 110 pN. Furthermore, the concentration of PilT retraction proteins did not affect the average stalling force. Thus, type IV pili are the strongest molecular motors reported to date, generating a 20-fold higher force than individual kinesin or muscle myosin molecules (Howard, 2001).

The question of whether pilus bundling can affect force generation has been addressed recently using a different assay. Biais *et al.* (2008) allowed gonococci adhere to micrometer-sized pillars, whereby each pillar acts as a force sensor that can measure forces in the nanonewton (10^{-9} N) range. They showed that single pilus retraction events were transient, similar to the laser tweezers assay. However, in the pillar assay, pili were able to form bundles consisting of up to 10 pili that attached to a pillar one by one. Bundles of pili generated and sustained forces in the nanonewton range, indicating that pili cooperated to generate and maintain extremely high molecular forces.

How robust is the ability of bacteria to generate a high molecular force by pilus retraction? The question of whether force generation was dependent on the architecture and surface properties of the pilus was addressed by measuring the stalling force in an *N. gonorrhoeae* strain in which the major pili subunit was replaced by the major pili from *Pseudomonas aeruginosa*. For pilin subunits of these strains, variations in the structure of several regions in the head domain and in posttranslational modification have been reported (Hegge *et al.*, 2004; Craig *et al.*, 2006). The pilin replacement did not significantly affect the stalling force, showing that force generation was robust against variations within the pilin subunit (Winther-Larsen *et al.*, 2007). The question of whether the ability to generate high force was during infection of their natural host, i.e. epithelial cells, was addressed in recent experiments (Opitz *et al.*, 2009). They revealed that although force generation was impaired (most likely due to elastic effects), forces in the range of *c.* 70 pN were generated for 24 h postinfection.

Control of direction and velocity of pili by mechanical force

In vitro single molecule experiments have revealed a general tendency of molecular motors to move at reduced velocity and increased probability for backward stepping when a high external force is applied (Bustamante *et al.*, 2004). Initial experiments with type IV pili seemed to show similar results as observations with motors *in vitro*. The average velocity decreased with increasing force and near the stalling force pili showed the tendency to switch the direction of movement and elongate (Maier *et al.*, 2004b). However, with an improved setup (with increased temporal resolution and force clamp), Clausen *et al.* (2009) showed that the dynamics of pilus length change depends on force in a more complex way. The histogram of retraction velocities showed a bimodal distribution, depending on the PilT concentration. This finding indicates that the pilus machine retracts

with two different molecular mechanisms. We speculate that the velocity mode may be determined by binding of one or more of the proteins that modulate pilus retraction mentioned in the previous paragraph. Switching between pilus retraction and elongation occurred at two different time scales (Fig. 3e and f). Switching at a time scale of seconds (Fig. 3e) is likely to be caused by binding and unbinding of the PilT hexamer shown in Fig. 3c. Switching at a time scale of milliseconds (Fig. 3e) may be attributed to back-stepping of the PilT hexamer. The probability for elongation decreased with the concentration of PilT per cell. Furthermore, the experiments shed new light on the interpretation of stalling of the motor at high forces (Clausen *et al.*, 2009). Although pili were unable to generate net movement near the stalling force of *c.* 110–120 pN, the average velocity of pilus retraction did not decrease at a high force, but the probability for pilus elongation increased. Based on these findings, we hypothesize that the external force most likely controls velocity and directionality through force-induced binding and unbinding of PilT and possibly regulating proteins.

Role of PilT oligomerization in type IV pilus dynamics

Presently, pilus retraction is too fast to resolve individual steps during pilus retraction, but the upper limit is 3 nm (Clausen *et al.*, 2009). Structural data suggest that the unit step size of pilus retraction is *c.* 1 nm, in agreement with removals of one pilin subunit from the pilus (Maier, 2005; Satyshur *et al.*, 2007; Craig & Li, 2008). The energy involved in removing a single pilin subunit from the pilus at 110 pN would be $E = 110\ \text{pN} \times 1\ \text{nm} = 110\ \text{pNnm}$. Most molecular motors have an efficiency of converting the energy yielded by ATP hydrolysis into a mechanical work of 20–100% (Bustamante *et al.*, 2004). Hydrolysis of one ATP *in vivo* yields only about 80 pNnm. Even if we assume 100% efficiency for pilus retraction, this estimation strongly suggests that the energy provided by the hydrolysis of a single ATP is not enough to power pilus retraction at high forces. The missing energy may be provided during polymerization and stored in the polymerized pilus. On the other hand, the application of uncouplers and ATP synthase inhibitors decreased the velocity of *N. gonorrhoeae* pilus retraction at low forces, but did not influence the average stalling force (Maier *et al.*, 2002). This observation suggests that ATP binding may be rate limiting at low forces and thus reduction of the ATP concentration decreases the rate at low forces. At a high force where the mechanical steps in the motor cycle become rate-limiting, the waiting time for binding of multiple ATP molecules may be negligible. These observations let to the hypothesis that hydrolysis of more than one ATP by the PilT hexamer may power pilus retraction at high external forces (Maier, 2005). The crystal structure of the PilT asymmetric hexamer indicated that two subunits simultaneously assume an active conformation and that structural changes within the ring are cooperative (Satyshur *et al.*, 2007) (Fig. 3c). The question of how coupling of multiple PilT molecules affects force generation and retraction velocity has been addressed in a theoretical model in which motors interact through deformations on a circular backbone with finite stiffness (Linden *et al.*, 2006). They found a regime in which the stalling force depended nonlinearly on the number of subunits. Furthermore, they reported that coupled motors supported pilus retraction more efficiently with a velocity vs. force relationship that was qualitatively different from individual PilT monomers. In summary, current data suggest that the ring-like structure of the PilT hexamer is important for its ability to generate high velocities even at a large external force, but future experiments are required to support this hypothesis.

DNA import during transformation

Functions of DNA transformation

Bacterial genomes often contain a large percentage of horizontally acquired DNA (Ochman *et al.*, 2000). DNA transformation is one of three mechanisms of horizontal gene transfer that enable bacteria to acquire new genetic information, including antibiotic resistance or virulence traits. Thus, competence for transformation can enhance the fitness of a bacterial cell population. Furthermore, DNA from related bacteria may serve as a template for DNA repair (Chen & Dubnau, 2004).

Competence for DNA transformation

During natural transformation, bacteria bind naked DNA to their cell surface and then transport DNA through the cell envelope. The newly acquired single-stranded DNA may be degraded, integrated into the chromosome by homologous recombination, or reconstituted in the case of self-replicating plasmids. In many bacterial species (e.g. *B. subtilis* and *Streptococcus pneumoniae*), competence for DNA transformation is a transient physiological state and the development is controlled by specific processes, including quorum sensing, nutritional signals, or antibiotics (Hamoen *et al.*, 2003; Claverys *et al.*, 2006; Leisner *et al.*, 2008). Some species (e.g. *N. gonorrhoeae* and *Neisseria meningitidis*) are constitutively competent throughout their growth cycles.

Various proteins are required for efficient DNA import by gram-positive bacteria, which can be subdivided into three categories: one group resembling type IV pilus/type II secretion proteins is required for DNA binding, one group

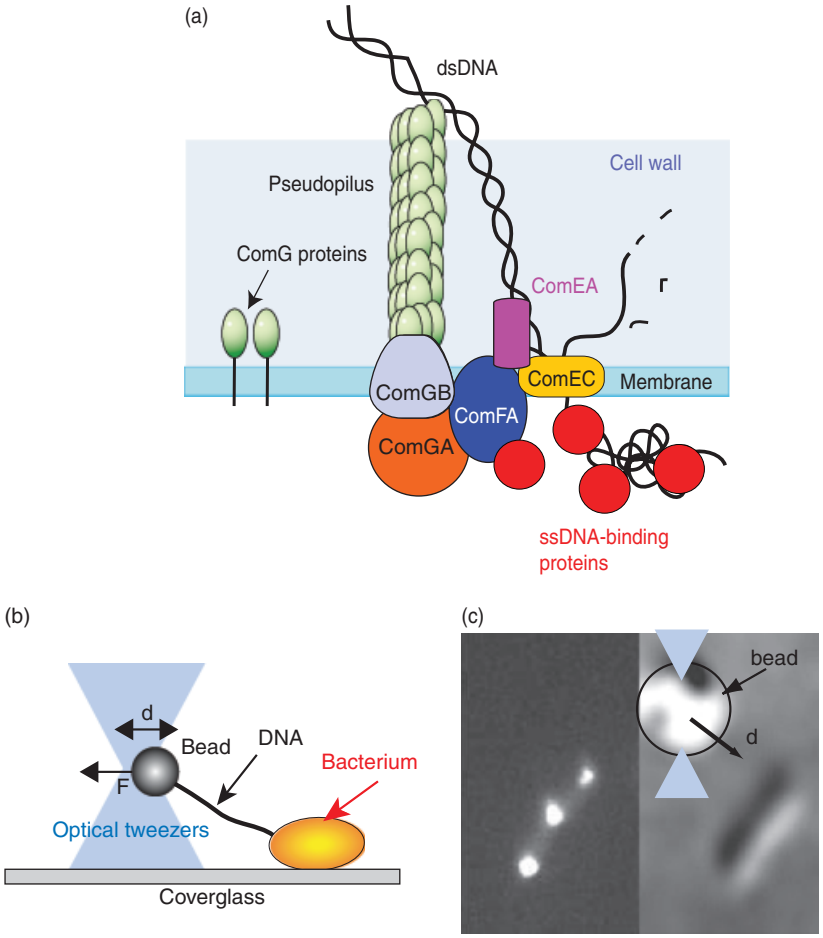

Fig. 4. Dynamics of the DNA import machine. (a) Basic elements of the DNA import machine. The putative molecular motors are ComFA, the ComG proteins forming the pseudopilus, or ssDNA-binding proteins. Please note that the full DNA import system consists of at least c. 20 proteins. (b) Sketch of a single molecule assay for studying DNA import. *Bacillus subtilis* binds to a glass surface. A DNA-coated bead in a laser trap is approached to the bacterium and upon binding of DNA to the bacterium the DNA tether is pulled straight. Once DNA import starts, DNA pulls on the bead and the latter is displaced from the center of the laser trap. (c) Laser tweezers were used to determine the location where DNA uptake occurred on the bacterium. Using a tracking algorithm, the directional bead movement was determined and extrapolated towards the cell (right). DNA uptake pulled the bead toward fluorescently labelled DNA import proteins (left), suggesting that polar DNA import complexes supported DNA translocation.

participates in DNA transport, and the last group consists of cytosolic proteins. In gram-negative bacteria, the outer membrane represents an additional barrier to DNA import. Most likely, DNA is transported through the outer membrane secretin PilQ (Assalkhou *et al.*, 2007; Collins *et al.*, 2004), but the mechanism that powers transport is unclear. In the following, we will focus on gram-positive bacteria and discuss the components of the DNA import machine using the example of *B. subtilis*.

The initial step to transformation is binding of double-stranded DNA to the cell surface. Binding is supported by ComG proteins that assemble the competence pseudopilus and by the membrane-bound protein ComEA (Provvedi & Dubnau, 1999) (Fig. 4a). NucA enhances the probability for DNA import through cleavage of dsDNA (Provvedi *et al.*, 2001). ComFA most likely (Londono-Vallejo & Dubnau, 1994a) supports DNA import through the putative channel protein ComEC (Draskovic & Dubnau, 2005) and cytosolic proteins (including SsbB, DprA, and RecA) bind to the newly acquired DNA in the cytoplasm (Berge *et al.*, 2003; Mortier-Barriere *et al.*, 2007; Tadesse & Graumann, 2007).

Dynamics and force generation during DNA import

Various experiments suggest that naturally competent bacteria assemble multiprotein machines for DNA import (Chen *et al.*, 2005). An important question was therefore at which rate individual DNA molecules were transported, whether transport was processive, and whether the machine could generate a mechanical force. Single molecule experiments are well suited to address these questions.

Maier *et al.* (2004a) have developed a method that allows measurement of the kinetics of the DNA transport machine in *B. subtilis* at the single molecule level (Fig. 4b). Bacteria were immobilized at a glass surface and approached a micrometer-sized DNA-coated bead using laser tweezers. Once a DNA molecule bound to the bacterium, it was stretched like a rubber band by moving the center of the tweezers away from the bacterium. Single molecule experiments suggest at least two different modes of binding; first, DNA was found to bind transiently to the bacterial cell surfaces and only a few of these binding events resulted in stable binding and import (Maier *et al.*, 2004a). When the

bacterium started to take up DNA, the position was detected at a resolution of 10 nm. The restoring force increased proportionally with the deflection of the bead from the center of the laser trap and the transport velocity against external force was measured. A highly processive molecular motor transported a DNA molecule with a length of 10 μm at a velocity of 80 bases s^{-1} and generated a force of at least 50 pN. Even at large counteracting forces, the DNA uptake motor did not reverse direction. Uncouplers inhibited DNA transport of individual DNA molecules immediately, indicating that transmembrane proton motive force is required for DNA translocation.

The DNA import machine

Single molecule experiments showed that DNA import is driven by a powerful and processive molecular machine. This observation is in very good agreement with recent experiments that support the picture of a large polar complex required for DNA import.

Representatives of membrane-bound proteins and cytoplasmic proteins accumulate and/or colocalize at the cell poles and accumulation is dynamic (Kidane & Graumann, 2005) (Hahn *et al.*, 2005). As competence develops, proteins accumulate and as transformability wanes proteins delocalize. Using the laser tweezers assay, the location of DNA uptake within a bacterium was determined by analyzing the direction at which bacteria attracted DNA-bound beads (Fig. 4c). DNA was taken up preferentially at the poles and uptake locations colocalized with foci of components of the DNA import machine (Hahn *et al.*, 2005). These experiments suggest that a whole machine transiently assembles for efficient DNA transport. To further support the picture of a multicomponent DNA transport machine, Kramer *et al.* (2007) used a combination of colocalization, FRET, and protein stability analysis to investigate interactions among DNA import proteins. They found that interactions occurred between membrane-associated proteins and cytosolic proteins, confirming the existence of a complex machine for binding, transport, and integration of transforming DNA.

Putative mechanisms that power DNA transport

Although a consistent picture of a large DNA import machine is emerging, it is currently unclear how the components interact to generate movement of the incoming DNA through the cell envelope. In the following, we will describe the mechanisms that have been proposed and discuss how the mechanisms can be addressed using single molecule techniques.

One proposed mechanism for driving DNA transport is pseudopilus retraction (Chen *et al.*, 2005) (Fig. 4a). The role of pseudopilus proteins in DNA import is very poorly understood. In *B. subtilis*, the pseudopilus is a complex build from the major pilins ComGC with an estimated length of 40–100 nm, that corresponds to the extension of the cell envelope in gram-positive bacteria (Chen *et al.*, 2006). Moreover, there is growing evidence in gram-negative bacteria (which generate pili whose length exceeds the cell envelope by far) that an alternative structure, also termed 'pseudopilus' in *B. subtilis*, may exist that competes for pilus components with elongated pilus fibers (Aas *et al.*, 2002; Long *et al.*, 2003). Repeated cycles of pseudopilus assembly and disassembly may drive DNA transport through the cell wall and through the pore in the cytoplasmic membrane. In this model, pseudopilus assembly would be powered by the (most likely hexameric) ATPase PilF in *Neisseria* or its orthologue ComGA in *B. subtilis*. Disassembly would then be driven by PilT in *Neisseria*. So far, no orthologue for PilT has been found in *B. subtilis*, but disassembly may occur either spontaneously or driven by proton motive force (Chen *et al.*, 2006). On the other hand, the pseudopilus may play a passive role and may form a structure through the cell envelope that permits the incoming DNA access to its membrane-bound receptor. At the single molecule level, the processes of type IV pilus retraction in *N. gonorrhoeae* and DNA import in *B. subtilis* exhibit strong similarities in terms of dynamics and force generation (Maier *et al.*, 2002, 2004a). With both processes, the translocation speed does not drop up to forces of 40 pN, which is highly unusual for molecular motors (Bustamante *et al.*, 2004). This observation suggests that pilus retraction and DNA transport may be driven by the same underlying mechanism. If cycles of pseudopilus assembly and disassembly would drive DNA import, pausing would be expected at 40–100 nm. Because the temporal resolution in previous experiments was 1 s (Maier *et al.*, 2004a), the rate of pseudopilus assembly would have to be $> 100\,\text{nm}\,s^{-1}$.

In gram-positive bacteria, ComFA is important, but dispensible, for DNA transport (Londono-Vallejo & Dubnau, 1994a, b). In ComFA knockout strains, DNA import proceeds at a 1000-fold reduced rate, strongly suggesting that ComFA supports DNA translocation, which may also occur at a lower rate through other mechanisms in its absence. ComFA is a membrane-bound ATPase that resembles DEAD box helicases. The latter are usually active as monomers or dimers (Cordin *et al.*, 2006). Titration experiments using ComFA with mutations in the putative ATP-binding site showed a dominant negative effect that suggests that more than one copy of ComFA is included in an active DNA translocation complex (Londono-Vallejo & Dubnau, 1994b). Furthermore, the fact that overexpression of ComFA has a detrimental effect on DNA uptake indicates that the correct stoichiometry within the DNA import machine is important for proper function (Londono-Vallejo & Dubnau, 1994a). No orthologue of ComFA has been identified in gram-negative bacteria (Chen & Dubnau, 2004). However, PriA

has sequence homology to ComFA, and PriA mutants were completely deficient in DNA transformation (Kline & Seifert, 2005).

A third mechanism for driving DNA import has been proposed, namely the passive mechanism of a translocation ratchet. Single strand-binding proteins could bias diffusion by binding to the incoming DNA in the cytoplasm and inhibiting backward diffusion (Fig. 4a). Using laser tweezers, Grange *et al.* (2008) recently demonstrated that a similar passive mechanism based on cooperative binding of VirE2 to ssDNA can generate a large molecular force that is likely to support DNA import in a type IV secretion system. Polymerization was detected at forces as high as 50 pN, but the polymerization rate declined exponentially even at low forces. Of particular interest in *B. subtilis* is SsbB (YwpH), which is annotated as a single strand-binding protein and colocalizes with other DNA import proteins at the cell pole (Hahn *et al.*, 2005; Kramer *et al.*, 2007). *ssbB* (*ywpH*) knockout mutants reveal a transformation efficiency that is decreased by a factor of only 10–50 as compared with the wild type (Lindner *et al.*, 2004). Other candidates for supporting a translocation ratchet would be DprA (Smf) or YjbF (CoiA) (Mortier-Barriere *et al.*, 2007; Tadesse & Graumann, 2007). Theoretical work predicts that variation of their concentration affects both force generation and kinetics (Peskin & Oster, 1995; Mogilner & Oster, 2003).

Conclusion and future prospects

Molecular translocation machines handle the difficult physical task of moving a substrate through a narrow constriction such as a pore or a closing septum. The single molecule approach has yielded important information about the physical properties of such translocation machines and provides a deeper insight into the molecular mechanism of the transport motors.

Interestingly, all four translocation machines discussed in this review generate very high molecular force exceeding 50 pN, and the type IV pilus motor is also the strongest linear motor reported so far. Furthermore the machines are bound to their substrates for long time periods depending on external force. What may be the function of high force generation? In the case of FtsK and SpoIIIE, it is very likely that a high force must be generated to displace DNA-bound proteins. Protein displacement by SpoIIIE has been directly demonstrated (Marquis *et al.*, 2008). A similar function of force generation can be envisioned for the DNA transport motor, because new DNA is likely to be acquired from lysed cells, although DNA is secreted by some species (Hamilton *et al.*, 2005). In the case of the pilus motor, force generation may support DNA import, but there is also evidence that force is a direct signal to host cells (Howie *et al.*, 2005).

All four processes transport their substrate along preferential directions. The common finding is that the reversal frequency of the transport direction increases with the external force. However, the mechanism that controls the direction is very different. Whereas FtsK and SpoIIIE read the chromosome polarity, the direction of pilus movement is most likely controlled by binding and unbinding of the motor protein, although this statement requires further experimental support. For DNA import during transformation, it is unknown how the direction of transport is determined.

Another common feature of FtsK, SpoIIIE, and PilT is that they form hexamers *in vitro*. Binding and hydrolysis of more than one ATP may provide the large amount of energy that is required for generation of a high force, and future experiments will have to confirm this speculation. Although there are indications of sequential ATP hydrolysis from structural studies, coordination between the subunits remains to be addressed by assessing whether individual subunits can support force generation similar to ClpX (Martin *et al.*, 2005) or whether the titration of one or multiple ATPase-deficient subunits interferes with the function of the whole hexamer as reported for ClpB (Werbeck *et al.*, 2008). Another important aspect of oligomerization is the cooperativity of ATP hydrolysis between the subunits. Cooperativity has been reported for certain ring-shaped oligomeric ATPases including for ClpX (Hersch *et al.*, 2005), but not for others, including the DNA packaging motor of bacteriophage Φ29 (Chemla *et al.*, 2005).

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