

# Visco-Elastic Membrane Tethers Extracted from *Escherichia coli* by Optical Tweezers

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**ABSTRACT** Tethers were created between a living *Escherichia coli* bacterium and a bead by unspecifically attaching the bead to the outer membrane and pulling it away using optical tweezers. Upon release, the bead returned to the bacterium, thus showing the existence of an elastic tether between the bead and the bacterium. These tethers can be tens of microns long, several times the bacterial length. Using mutants expressing different parts of the outer membrane structure, we have shown that an intact core lipopolysaccharide is a necessary condition for tether formation, regardless of whether the beads were uncoated polystyrene or beads coated with lectin. A physical characterization of the tethers has been performed yielding visco-elastic tether force-extension relationships: for first pull tethers, a spring constant of 10–12 pN/ $\mu$ m describes the tether visco-elasticity, for subsequent pulls the spring constant decreases to 6–7 pN/ $\mu$ m, and typical relaxation timescales of hundreds of seconds are observed. Studies of tether stability in the presence of proteases, lipases, and amylases lead us to propose that the extracted tether is primarily composed of the asymmetric lipopolysaccharide containing bilayer of the outer membrane. This unspecific tethered attachment mechanism could be important in the initiation of bacterial adhesion.

## INTRODUCTION

Tethers have been reported extracted from artificial vesicles, blebbing cells (1–5), and living eukaryotic cells (6–12). However, to our knowledge, this is the first study addressing the extraction of tethers from prokaryotic organisms, the membrane properties of which are of extreme importance for the study of antibiotics. In the case of tethers extracted from vesicles, the theory describing the process is well developed and the systems fairly well understood (1–5). In the case of the eukaryotic cells, the force necessary to pull a tether from the membrane must overcome the bending rigidity of the membrane, the viscous resistance of the phospholipid bilayer, and the adhesion of the bilayer to the cytoskeleton. The force-extension relations can be correspondingly characterized; in the pre-tether phase, a pointlike force will first deform the cell, causing a rapid increase in force, then the force pulls the bilayer away from the cytoskeleton and as soon as that happens, the force decreases. After this, a tether can be extracted by a fairly constant force which is lower than initially needed to deform the cell and initiate tether formation (4,10,13). In the constant force region, lipid is anticipated to flow from a membrane reservoir into the tether (1,6).

We have extracted elastic membrane tethers from *Escherichia coli* by unspecifically attaching beads to the outer membrane structure, and then used the beads as handles for optical tweezers which, while pulling a bead, exerted a pointlike force on the outer membrane. Apart from a manipulation

tool, the optical tweezers also served as a detection method capable of measuring corresponding values of forces and extension.

The Gram-negative *E. coli* bacterium has a multilayered wall that envelopes its cytoplasm. The outer membrane is a lipid bilayer with a highly asymmetric distribution of the lipids (14,15). The bilayer contains two types of lipids: lipopolysaccharides (LPS) and phospholipids. The outer leaflet consists mostly of LPS and the inner leaflet consists mostly of phospholipid, the major phospholipid types being phosphatidylethanolamine (70–80%), phosphatidylglycerol (15–25%), and cardiolipin (5%) (14). Because of the strong interaction among the LPS molecules, it constitutes a barrier difficult to penetrate for various proteins and molecules in comparison to the inner phospholipid layer (14). Passage of nutrients and water through the LPS leaflet mainly happens through specific channels. Beneath this highly asymmetric LPS-phospholipid bilayer in the periplasmic medium is the peptidoglycan layer. This has a very rigid structure, allowing for large osmotic pressure differences across it, and gives the rodlike shape to the bacterium. The most inner part of the cell wall structure is a lipid bilayer which resembles the lipid bilayer surrounding eukaryotic cells.

Fig. 1 shows the LPS layer which is unique for the outer membrane of Gram-negative bacteria. The layer consists of three structurally and functionally different domains: the innermost lipid A, the inner and outer core part, and the o-antigen, which again consists of repeating sugars that reach out into the extracellular medium. Different bacterial strains express different types of LPS; in our study we have employed so-called smooth, rough, and deep rough chemotypes. The smooth chemotype has intact o-antigens. The rough strain lacks the o-antigen part, but does have intact

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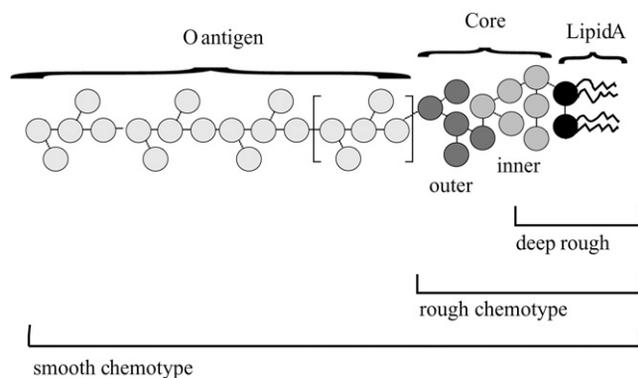


FIGURE 1 A rough sketch of a lipopolysaccharide (LPS) of a Gram-negative bacteria. From left (*outside*) to right (*inside*) a LPS molecule consists of an o-antigen part linked together by glucosidic bonds, a core region divided in an inner and outer region, and the lipid A. Parts of LPS expressed in the different chemotypes are also shown.

core LPS. The LPS layer of the deep rough strains is stripped down and only expresses the inner part of the core and lipid A. In the outer membrane of *E. coli* bacteria there exists a range of extracellular organelles and some of the investigated strains also express pili. Pili are a kind of fimbriae used by some bacterial strains to mediate adhesion to the host strain or to exchange genetic material. However, the observed tethers in this study are not pili, since their force-extension characteristics are totally different (16), and as one of the used strains does not express pili but nevertheless creates tethers.

Our results revealed that an intact LPS core is crucial for tether formation. Force-extension measurements demonstrated that the tethers have both elastic and viscous properties depending on the timescales involved. Also, it is clear that the tethers extracted from bacterial outer membranes were very different in nature from the tethers extracted from vesicles or eukaryotic cells. Finally, by investigating the sensitivity of the tethers toward a variety of enzymes, we put forward a model of the constituents of the observed bacterial tethers.

## MATERIALS AND METHODS

### Bacterial strains

The two rough chemotypes employed are the host strains CS180 (17) and S2188 with the inserted plasmid pSB2267 (18). The smooth strain CS1861 and the deep rough strain CS2429 (17), which are both substrains of CS180, are also used. The S2188 strain is grown as described in Oddershede et al. (19). CS180 and its substrains are grown in a similar way but in a rich LB-media instead of the minimal M63 media.

### Microspheres

The beads were polystyrene beads from Bangs Laboratories (Fishers, IN) with a diameter of 1.01  $\mu\text{m}$ . These beads were washed by suspending 25  $\mu\text{L}$

beads in 975  $\mu\text{L}$  Millipore water (Millipore, Billerica, MA) and centrifuging at 4000 rounds per min for 4 min. The supernatant was discarded and the pellet resuspended in 50  $\mu\text{L}$  PBS buffer and put in an ultrasonic bath for 10 min. For some of the experiments the beads were coated with lectin. To coat the beads, the solution was mixed with 10  $\mu\text{L}$  bovine serum albumin (BSA) solution (10 mg/mL) and 10  $\mu\text{L}$  lectin solution (0.2 mL wheat germ agglutinin in 1 mL 25 mM bicarbonate buffer pH 8, 0.9% NaCl, 2 mg/mL BSA). The bead solution was oscillated for 2 h at a temperature above 20°C, then the beads are washed three times as described above, but in PBS buffer instead of Millipore water. Finally, the beads are suspended in 200  $\mu\text{L}$  PBS buffer.

### Perfusion chambers

Perfusion chambers were made as described in Oddershede et al. (19), where the M63 media is interchanged with LB media when using the strains CS180, CS1861, and CS2429. After the bacteria had been allowed to settle to the poly-L-lysine coated surface, beads were flushed in and incubated with the bacteria for 20 min. After this, the chamber was washed with buffer, thus removing any beads that were not attached. One of the beads which had adhered unspecifically to the bacteria was optically trapped and pulled away from the bacteria, in this way creating a tether between the bead and the bacterium.

### Enzymes

The enzymes employed were the lipase *LIPOPAN F*, the amylase *TERMAMYL*, and the protease *SAVINASE*, all of which were kindly provided by Novozymes A/S (Bagsværd, Denmark). They are described in detail below:

The lipase is a hydrolase with specificity toward both phospholipids and triglycerides. Therefore, if there are no limitations on the diffusion, the enzyme is anticipated to attack the innermost bilayer of the *E. coli* cell wall and potentially also the glucosamine-based phospholipid lipid A in the outermost leaflet of the lipid bilayer.

The amylase cleaves polysaccharides by hydrolyzing long chains of carbohydrates; the products are smaller sugar units. The amylase has specificity for long sugars with  $\alpha$  1,4 glycosidic bonds as, e.g., the o-antigen. Calcium is part of its structure and is a cofactor necessary for the functioning of the enzyme.

The protease hydrolyzes peptide bonds between amino acids of proteins. This protease is a serine protease and member of the subtilisin family, which is the second largest serine protease family. This protease has specificity against a large range of proteins, i.e., supposedly also pili proteins and the proteins embedded in the membrane.

All enzymes are flushed into the perfusion chamber in a concentration of 1  $\mu\text{M}$  in a KCl-potassium phosphate (10 mM potassium phosphate, 0.1 M KCl, pH 7) buffer with 0.1 mM  $\text{CaCl}_2$ . Control measurements without the above enzymes are done with a 1  $\mu\text{M}$  bovine serum albumin (BSA) solution. These conditions are the optimal conditions for the enzymes, as investigated by Novozymes. The enzymes are incubated for at least 10 min with the bacteria before the tethers are pulled and the enzymatic experiments initiated.

### Optical tweezers

Our optical tweezers setup is based on an Nd:YVO<sub>4</sub> laser with a wavelength of 1064 nm. It is capable of measuring corresponding forces and distances in the picoNewton and nanometer regimes with a time resolution of microseconds using a quadrant photodiode system for detection (20,21). Furthermore, a piezoelectric stage is used to move the specimen relatively to the optical tweezers.

## Force measurements

The optical tweezers are exerting a harmonic force on the bead in the trap,  $F_{\text{trap}} = \kappa_{\text{trap}}x_{\text{trap}}$ , where  $\kappa_{\text{trap}}$  is denoted the trap stiffness and  $x_{\text{trap}}$  is the position of the bead with respect to the center of the trap. By measuring the positions visited by an optically trapped bead performing Brownian motion,  $\kappa_{\text{trap}}$  is found (20). This was done in every single perfusion chamber under the same conditions as the experiments (e.g., with the same distance to surfaces). We utilized the routines described in Oddershede et al. (22) and the software described in Hansen et al. (23) for the calibration procedure, this software also taking into account, e.g., the filtering effect of the quadrant photodiode (21). To obtain the total extension of the tether,  $x$ , the voltage signal giving the position of the piezoelectric stage  $V_s$  and the corresponding signal from the photodiode  $V_x$ , giving the position of the bead relative to the center of the trap, are analyzed as

$$x = B \times V_s - x_{\text{trap}} - r - x_0, \quad (1)$$

where the first term describes the distance that the stage has been moved,  $B$  being the conversion that translates the voltage signal from the piezoelectric stage to metric coordinates. The third term  $r$  is simply the radius of the bead as the extension,  $x$ , is measured as the distance from the surface of the bacterium to the surface of the bead. However, the starting point is arbitrary and therefore some constant,  $x_0$ , is subtracted. In practice,  $r$  and  $x_0$  are merged into a single fitting constant.

The total force,  $F$ , acting on the tethered bead, is found as

$$F = \kappa_{\text{trap}} \times x_{\text{trap}} - \gamma \times v, \quad (2)$$

where  $v$  is the velocity of the fluid relative to the sphere, which equals the velocity of the stage and  $\gamma$  is the friction coefficient. The last term is the Stoke's drag on the sphere, which is found to be approximately four orders-of-magnitude smaller than the first term and hence safely can be neglected. As the zero point of the force is somewhat arbitrary, the measured forces are relative, not absolute.

## RESULTS

The tethers were not readily visible with bright field microscopy; however, the fact that the bead could only be pulled a certain distance away from the bacteria, and the fact that the bead returned to the bacterial body upon release from the optical trap, proved that a tether was indeed present (see Supplementary Material movie). The inset in Fig. 3 shows a sketch of the experiment. We tried to visualize the tethers using DIC microscopy, which has been used to visualize, e.g., tethers extracted from blebbing cells (2). Also, we tried to visualize the tethers by fluorescence, as, e.g., done for outer hair cells (10), by staining the lipids by Nile red. However, both these attempts were not successful, maybe because the radius of the prokaryotic tethers is significantly smaller than the tether types henceforth reported. We also pulled tethers with larger beads having a diameter of  $\approx 2 \mu\text{m}$ , but still the tether remained invisible.

### Tether formation dependence on chemotype

To investigate which parts of the outer membrane structure are forming the observed tethers we tried to extract tethers from smooth, rough, and deep rough chemotypes. An overview of the used chemotypes is given in Table 1.

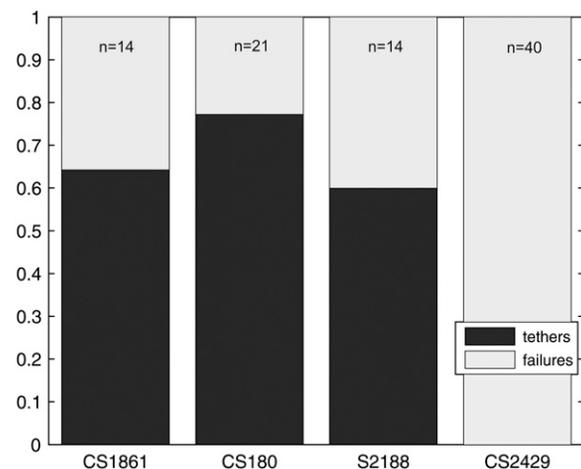
**TABLE 1** Overview of names and chemotypes of the employed strains as well as references to where they are first described

Name	Chemotype	References
CS1861	Smooth	(17)
S2188	Rough	(18,19)
CS180	Rough	(17)
CS2429	Deep rough	(17)

The experiment was done by letting beads unspecifically adhere to the surface of the bacteria for  $\sim 20$  min and then pull them away using the optical trap at a constant velocity. A certain fraction of the trials resulted in successful tether formation between the bead and the bacterium. The normalized fractions of successful tether formations for the various chemotypes are shown in Fig. 2. Tethers were easily pulled from the smooth and rough strains; however, from the deep rough strain no tethers could be extracted. A Student's  $t$ -test shows that there is no significant difference in the ability to extract tethers from smooth and rough chemotypes. Similar results are obtained whether uncoated polystyrene beads or beads coated with lectin are used. As tethers cannot be extracted from the deep rough strains lacking the inner part of the core and the lipid A, but can be easily extracted from the smooth and rough strains, it seems likely that an intact core LPS is crucial for tether formation but the o-antigen part need not be intact.

### Tether visco-elasticity

Typical force-extension relations for a tether extracted at constant velocity and forced to relax at the same speed are shown in Fig. 3. In general, the slope of the curve is dependent on the number of pulls the tether has been exposed



**FIGURE 2** This figure shows from which of the strains tethers could be created successfully. The fraction of pulls resulting in the creation of tethers is labeled *tethers*. The number of pulls that did not create tethers is labeled *failures*. The value  $n$  is the total number of experiments of one column. From left, the bars show the distributions for the smooth CS1861, the rough CS180, the rough S2188, and the deep rough CS2429 strains.

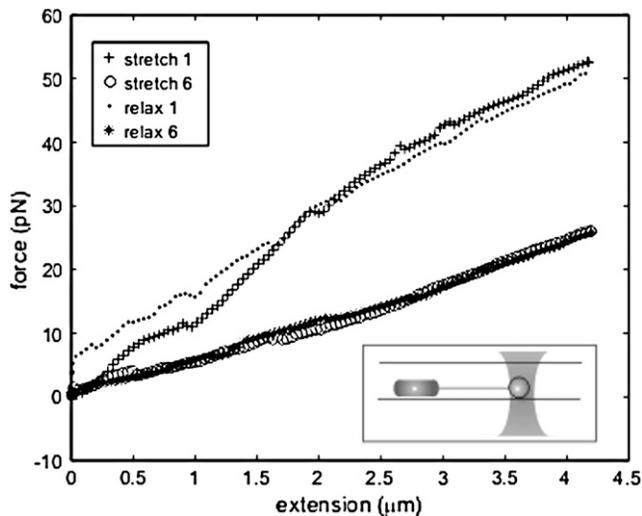


FIGURE 3 Force-extension relations for two cycles of *stretch* and *relax* of a tether extracted from S2188 at a constant velocity of  $0.2 \mu\text{m/s}$ . The upper *stretch1* and *relax1* are the curves for the first time the tether is stretched and relaxed. The labels *stretch6* and *relax6* are curves resulting from the sixth cycle. Inset shows a sketch of the experiment.

to; Fig. 3 shows traces both from a first and a sixth pull of a given tether. Overall, the relation between force and extension obeys Hooke's law:  $F = \kappa x$  where  $F$  is the force,  $x$  is the extension, and  $\kappa$  is an effective spring constant describing the elasticity of the tether. Supplementary Material Fig. 7 shows how  $\kappa$  decreases with number of pulls for a given tether and finally reaches a nearly constant value. The value of  $\kappa$  is found by a linear fit to the region from  $0.5$  to  $3 \mu\text{m}$ . For 15 first-pull tethers the value of obtained spring constants  $\kappa_{\text{S2188}}$  is found to be  $(11.6 \pm 3.5) \text{ pN}/\mu\text{m}$  (mean  $\pm$  SD) for tethers extracted from the rough S2188 strain. For 17 tethers extracted from the smooth CS1861 strain,  $\kappa_{\text{CS1861}} = (9.9 \pm 3.0) \text{ pN}/\mu\text{m}$ . Within the uncertainties, these values describing the effective spring constants of the two strains are identical. After successive pulls, the spring constants relaxes to constant and significantly lower values,  $(5.7 \pm 1.2) \text{ pN}/\mu\text{m}$  for the rough S2188 and  $(7.3 \pm 3.7) \text{ pN}/\mu\text{m}$  for the smooth CS1861—the numbers from the different types of strains being identical within the uncertainties.

The experiments were done at a pulling rate  $0.2 \mu\text{m/s}$ . This indicates that, at short timescales ( $\leq 10 \text{ s}$ ), the tether appears elastic, but on longer timescales (during the consecutive pulls) a viscous relaxation occurs, thus decreasing the apparent spring constant. This viscous relaxation is probably due to a relocation of membrane material. Results from experiments done at pulling velocities of  $0.1 \mu\text{m/s}$  were indistinguishable from those done at  $0.2 \mu\text{m/s}$ . If the experiment was done slower than  $0.1 \mu\text{m/s}$ , then drift became a problem. If the experiment was done faster than  $0.2 \mu\text{m/s}$ , the bead would escape the trap unless laser power was ramped up significantly, thus increasing the risk of optically damaging the sample.

The viscous properties of the tethers were further investigated by an experiment where a tether was first pulled out as usual, but then the stage was halted at its extreme position and the force acting on the bead was monitored as a function of time. Fig. 4 shows the result of such an experiment, where the tether was pulled out in the first 40 s and then it was left to relax for an additional 350 s. The decay in the tethering force is exponential, consistent with the observations of tethers from red blood cells (7), from vesicles (1,24), and from human neutrophil cells (12). This type of decay is called a Maxwell-like decay and is a typical sign of visco-elastic systems. To find the characteristic relaxation time,  $\tau$ , the decay was fitted by

$$F = \alpha \times \exp\left(-\frac{t}{\tau}\right) + \beta, \quad (3)$$

where  $t$  is time,  $\alpha$  is the total force decay, and  $\beta$  is equilibrium tethering force which is asymptotically approached. This rendered a relaxation time of  $\tau = (207 \pm 127) \text{ s}$  (mean  $\pm$  SD). The equilibrium force asymptotically approached was  $\beta = (17.7 \pm 2.4) \text{ pN}$ .

### Enzyme sensitivity of tethers

To address the biochemical composition of the tethers, extracted tethers were exposed to the enzymes described in Materials and Methods and summarized in Table 2. First, the bacteria were incubated with the enzymes for at least 10 min and then tethers were extracted from smooth or rough bacteria in the presence of the enzymes. The condition employed to determine the sensitivity of a tether was whether or not the tether had been cut within the first 60 seconds after its extraction. Each of the enzymes was probed separately. Also, we tried a combination of all enzymes simultaneously as well

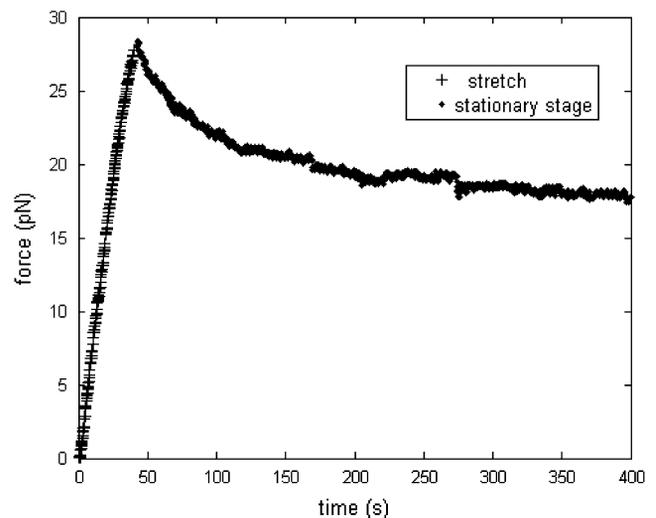


FIGURE 4 Within the first 40 seconds, a tether is extracted from the rough strain S2188 to a distance of  $4 \mu\text{m}$  with a velocity of  $0.1 \mu\text{m/s}$ . At this extreme position the stage is stopped and the figure shows how the force in the tether relaxes as a function of time.

**TABLE 2 Overview of the various enzymes employed, their name, specificity, and putative targets in the outer membrane of *E. coli* bacteria**

Enzyme	Specificity	Targets in the <i>E. coli</i> envelope
Lipase	Acyl ester bonds in phospholipids and glycerides	Phospholipids and lipid A
Protease	Peptide bonds	Membrane proteins, pili-proteins
Amylase	$\alpha$ 1,4 glycosidic bonds	o-antigen

as a control containing no enzymes but an equivalent concentration of BSA. Fig. 5 shows the effect of the enzymes on the stability of the tethers. Student's *t*-tests show that there is no significant effect of the protease on tethers extracted from the smooth CS1861 or the rough S2188. There is a significant effect of the lipase on tethers extracted from the rough S2188 strain, but no significant effect of the lipase on the smooth chemotype CS1861. The reason for the difference in sensitivity of the two chemotypes could be that the presence of intact o-antigens in the smooth chemotype hinders the lipase in reaching its target, lipid A. For the rough chemotype, the lipase more easily penetrates to lipid A, thus destabilizing the tether.

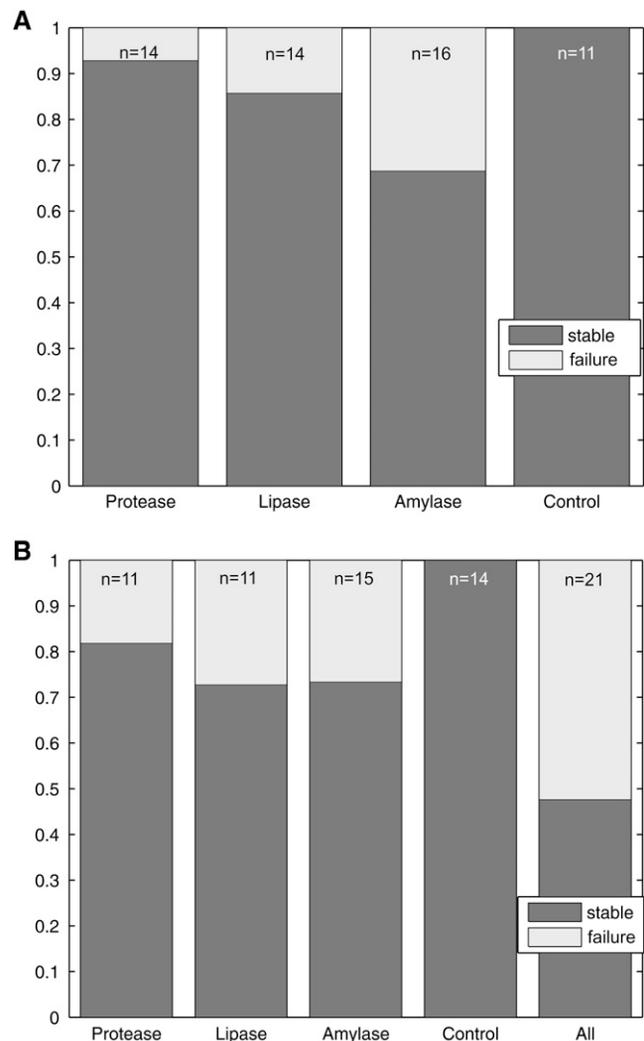
Tethers extracted from both the smooth CS1861 and the rough S2188 are seen to be destabilized by amylase which hydrolyzes the glucosidic bonds in the LPS. This indicates that not only the o-antigen is disrupted by the amylase but other parts of the LPS as those present in the rough chemotype are affected by the amylase, potentially in the peptid-o-glycan core. When all enzymes are present simultaneously, more tethers are cut than when probing either of the enzymes individually. The controls all have tethers which are stable for >60 s.

Before the experiments enzyme activity had been checked by Novozymes A/S. The fact that the tethers were sensitive to the attack of amylase and lipase proved that these enzymes were also active under the experimental conditions. To check the activity of the protease under the experimental conditions, we conducted an experiment where the protease was mixed with 3 mg/mL BSA under the same conditions as the tether experiments and the change in pH was followed. A significant change in pH showed that the hydrolyzing protease was active.

As another control measurement we measured the effects of the enzyme solutions with and without calcium. We found that the actions of the protease and the lipase are independent of the presence of calcium in the solution. However, the activity of amylase is crucially dependent on the presence of calcium, as expected.

## DISCUSSION

From the experiments based on different *E. coli* chemotypes (see Table 1) with differences in the intactness of the outer



**FIGURE 5** Sensitivity of tethers toward various enzymes. The value *n* is the total number of experiments of a given kind. (A) Stability of tethers from the smooth mutant CS1861 in the presence of protease, lipase, or amylase. The control experiment does not contain any enzymes but BSA in an equivalent concentration. (B) Stability of tethers from the rough mutant S2188 in the presence of protease, lipase, or amylase. Again, the control only contains BSA. The last column shows the sensitivity of the tether when all enzymes are present.

membrane structure, we have shown that tethers can only be extracted from strains having an intact LPS core. No difference in ability to produce tethers was found between mutants with an intact LPS and the rough mutants that lack the o-antigen part. These results are independent of whether the polystyrene bead was coated with lectins. Hence, formation of stable tethers cannot be explained by a specific binding between the lectins and the LPS.

The force necessary to pull the bacterial tethers increased linearly with tether elongation. The tether stiffnesses for first pulls were found to be  $(11.6 \pm 3.5)$  pN/ $\mu$ m (mean  $\pm$  SD) for tethers extracted with 0.2  $\mu$ m/s from rough S2188 and

( $9.9 \pm 3.0$ ) pN/ $\mu\text{m}$  for smooth CS1861. After successive pulls, the spring constants were observed to relax to the constant and significantly lower values ( $5.7 \pm 1.2$ ) pN/ $\mu\text{m}$  for the rough S2188 and ( $7.3 \pm 3.7$ ) pN/ $\mu\text{m}$  for the smooth CS1861. The relaxation of the effective spring constant with number of pulls has not been described for other systems in the literature. The relaxation probably reflects how the viscous properties of the tether adjusts to the extension process. The linear relation between force and elongation of the bacterial tethers is different from the relations reported for most eukaryotic and vesicle systems (see, e.g., (4,10,13)). The normal force-extension behavior of a eukaryotic or vesicle tether is that first, the force increases to a peak value often denoted the tether force, which is needed for pulling a tether visibly away from the cell. When pulling is continued after this peak force value, the force necessary to elongate the tether is nearly constant and sometimes significantly lower than the peak value. Only in a study of fibroblasts (13) did they report a nearly linear force-elongation relation for the initial part of the elongation, yielding a stiffness of  $\sim 6$  pN/ $\mu\text{m}$ , a number comparable to the our observations from prokaryotic tethers.

The prokaryotic tethers are typically stable for hundreds of seconds and have linear force-extension relations over distances that are several times the bacterial length. In the microscope, absolutely no deformation of the bacterial shape is visible.

The bacterial tethers are not purely elastic, for they also show viscous behavior: When allowed to relax at a constant extension, the tether force decreased exponentially to a constant value  $\sim 60\%$  of the peak value. The exponential decaying time was  $\tau = (207 \pm 127)$  s. This number can be compared to relaxation times of  $\sim 1$  s for a human neutrophil (12), 50 s for an outer hair cell tether (10), 86 s for a vesicle (1), and  $\sim 250$  s for red blood cells (7). Hence, the relaxation timescale found for a prokaryotic tether falls within the previously observed interval, though closest to the value observed for tethers extracted from red blood cells. As the force of a taut tether relaxes, most likely material is flowing from the membrane into the tether (1,25).

The tethers originating from the smooth and rough chemotypes were sensitive to the action of amylase. This shows that LPS is a crucial part of the extracted tethers. The smooth and rough chemotypes have different sensitivities toward the lipase, the smooth being insensitive to the enzyme, and the rough being sensitive. This is probably because, for the smooth chemotype, the presence of an intact o-antigen makes it difficult for the lipase to penetrate into the outer membrane structure and attack the lipid components, including phospholipid and lipid A. The rough chemotype, on the other hand, lacks the o-antigens, so the lipase is able to gain access into the membrane structure and hydrolyze the lipid components and hence make the tether unstable. Both chemotypes are insensitive to the protease, thus showing that proteins either do not extend out in the tether (e.g., remain

connected to the peptid-o-glycan layer) or that the intactness of proteins in the tethers is not crucial for the tether stability.

On the basis of the above observations we propose a model for the bacterial tethers. As sketched in Fig. 6, we suggest that an unspecific bacterial tether consists of a double layer of lipopolysaccharides and phospholipids. The fact that tethers cannot be extracted from the deep rough strains suggests that the core part of the LPS is crucial for tether formation; possibly the core stabilizes the tether. However, an intactness of the o-antigen is not necessary for tether formation. The stiffness of an isolated hydrated peptid-o-glycan layer has been measured by AFM in a direction orthogonal to the layer, yielding an elastic modulus of  $2.5 \times 10^7$  N/m<sup>2</sup> (26); therefore, it is very unlikely that we are able to pull it out using forces of only up to 50 pN. Hence, we do not believe the peptid-o-glycan layer is part of the tethers. This is consistent with the fact that no deformation of the cell shape is observed upon pulling the tethers. Also, we do not expect that any parts beneath the peptid-o-glycan layer are part of the tethers. In other words, we consider the tethers to be a physical property of the *E. coli* outer membrane. Supporting our model, in a similar fashion, we tried to extract tethers from Gram-positive *Bacterius subtilis* organisms, which are not surrounded by an outer membrane. In 19 trials no successful tether was formed thus supporting our model that the tethers mainly consist of outer membrane material.

Based on the model, we suggest that the force needed to extract elastic bacterial tethers has at least five constituents: The force needed to overcome the adhesion of the outer

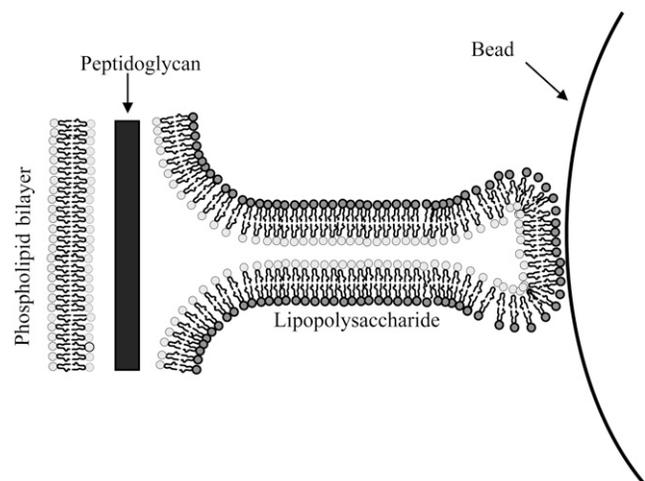


FIGURE 6 Proposed model of the observed visco-elastic bacterial tether stemming from a rough bacterial strain. The tether spanning the distance between the bead and the body of the bacterium is proposed to consist of the asymmetric LPS and phospholipid bilayer but not of the peptid-o-glycan layer or anything beneath it. Possible membrane proteins are not thought to play any crucial role and, hence, are not depicted. The o-antigen part is not drawn on this picture, and for tethers extracted from smooth strains, this should be envisioned too.

membrane to the peptidoglycan layer; the viscous drag of the LPS containing bilayer as it is withdrawn; the frictional force between the two leaflets (1,25) (this force probably being larger for a prokaryotic membrane with a large chemical difference between the two leaflets than for, e.g., tethers created from a vesicle); the force necessary to bend the withdrawn part of the membrane; and the Stoke's drag force on the microsphere (as discussed earlier, the latter contribution being negligible).

To our knowledge, neither the observed tethers nor their physical and biochemical characteristics have yet been described in literature. We wish to emphasize that the observed tethers are not bacterial pili, which are well described in literature. One argument for this is that one of our tether-producing strains, the rough S2188, does not express any pili proteins—it is *fim::kan* (18). Another argument is that the forces needed to extract pili a distance of a micron are  $\sim 50$ – $100$  pN (16), whereas the bacterial tethers here observed can be pulled a few microns out using forces only at  $\sim 10$  pN. Also, the force extension curves outline is significantly different (which can be seen by comparing Fig. 3 to Fig. 7 or 8 from (16)). Finally, the tethers are not sensitive to the proteases which are likely to attack pili.

As the tethers of this study were created by a nonforced nonspecific adhesion of beads to bacteria during tens of minutes, it is likely that this adhesion process resembles the first stages of bacterial adhesion either to other microscopic particles or to host organisms. We propose that the very first attraction between bacteria and beads is through the van der Waals interaction and that the bacteria respond to the vicinity of a bead by making unspecific bonds to the bead. Bonds are strong enough that a tether consisting of the LPS containing bilayer can be extracted from the bacteria upon removal of the bead. We believe that the unspecific bacterial tethers observed here could be a part of the very first unspecific attachment process between the bacterium and any solid support. The bacteria can then later anchor themselves more permanently using cell adhesion molecules such as pili-proteins or other specific protein interactions.

## CONCLUSION

We have studied tether formation from living *E. coli* bacteria and performed a physical as well as a biochemical characterization of these tethers. A bead, either uncoated or coated with lectin proteins, was attached to the outer membrane of an *E. coli* bacterium and pulled away from the organism using optical tweezers, thus creating a viscoelastic tethering between the bead and the bacterium. Tethers could only be extracted from chemotypes expressing an intact core in the lipopolysaccharide layer, this being a crucial part of the observed unspecific tethers. The force-extension curves obey Hookean behavior, proving an elastic nature of the tethers. However, the effective spring constants changed with the number of times a particular tether was pulled: A first pull

tether typically had a spring constant of  $10$ – $12$  pN/ $\mu\text{m}$  for both rough and smooth chemotypes, but for consecutive pulls the effective spring constant relaxed to a value of  $6$ – $7$  pN/ $\mu\text{m}$ . This change of effective spring constant is probably due to a viscous relaxation of the tether, this relaxation also manifesting itself in an exponential decay of the force necessary to hold a tether taut. The tethers proved to be sensitive to a carbohydrate-degrading enzyme which putatively targets the o-antigen part of the lipopolysaccharide structure. The rough chemotype, lacking the outer part of the o-antigen, was also sensitive to a lipid-degrading enzyme, which may attack the inner part of the lipopolysaccharide layer, including the lipid A. However, the smooth chemotype with an intact o-antigen was not as sensitive to lipase, probably because the lipase was hindered in reaching lipid A. Based on these observations, we propose that the tethers consist of the asymmetric lipopolysaccharide-containing bilayer, but the very stiff peptid-o-glycan layer, and anything beneath it, is not part of the tether.

These unspecific tethers that living bacteria are able to produce might have important tasks in the very initial stages of bacterial adhesion to solid supports. The tethers are clearly different than, e.g., bacterial pili and have, to our knowledge, not been described before. The results presented here open the route to many other questions concerning bacterial unspecific tethering, e.g., the exact purpose of the mechanism, or a more precise understanding of the contribution of the different molecules and forces in play.

## SUPPLEMENTARY MATERIAL

To view all of the supplemental files associated with this article, visit [www.biophysj.org](http://www.biophysj.org).

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