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Interaction of the N-terminal domain of MukB with the bacterial tubulin homologue FtsZ

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Abstract The MukB protein is involved in the process of chromosome partition in *Escherichia coli* and has a domain structure reminiscent of the eukaryotic motor proteins kinesin and myosin. This has led to the suggestion that MukB may function as a motor protein in vivo. In order to test this idea we have recombinantly expressed the N-terminal domain of MukB (residues 1–342) as a poly-His tagged fusion protein for biochemical characterisation. The purified protein (Muk342) is monomeric and has low basal Mg-ATPase (1.23 min^{-1}) and Mg-GTPase (0.17 min^{-1}) activities. Muk342 binds with high affinity to the prokaryotic tubulin homologue FtsZ and we have evidence that FtsZ can stimulate nucleotide turnover by Muk342. These properties are consistent with MukB functioning as a motor protein using FtsZ as a track or anchor for generating force within *E. coli*.

Key words: Chromosome partition; FtsZ; Motor; MukB; Tubulin

1. Introduction

The MukB protein is a large multi-domain protein (M_r = 177 kDa) involved in the process of chromosome partition in *Escherichia coli* [1]. During chromosome partition the replicated nucleoids are accurately positioned at the one quarter and three quarter cell lengths of the bacterium before it divides into two daughter cells. The gene for MukB was identified from a genetic screen of partition deficient strains as being defective in the positioning of replicated chromosomes [1]. The protein has not been characterised biochemically in any detail. However, consistent with its proposed role in chromosome movement, an earlier study demonstrated that it has a DNA binding activity and could be crosslinked with either ATP or GTP in the presence of Zn^{2+} ions [1,2].

MukB shares a number of similarities with members of both the kinesin and myosin superfamilies (Fig. 1). For example, it forms homodimers and has twin N-terminal globular domains followed by a region of predicted coiled coil which links it to C-terminal globular domains [1,2]. The N-terminal globular domain is about the same size as that of the kinesin motor domain (~340 residues) and contains nucleotide binding motifs [1].

If MukB functions as a motor protein then this N-terminal domain might be expected to share a number of properties with the motor domains of the eukaryotic motors, kinesin and myosin. Firstly, it should hydrolyse nucleotides such as ATP or GTP to provide the energy for movement. Secondly, it should interact with proteins capable of polymerising into filaments and acting as a track (e.g. prokaryotic equivalents of either tubulin and actin). Thirdly, it should show a nucleotide dependence in its interaction with the polymerised track protein. Obviously as final proof of motor protein activity it would be nice to provide evidence that the protein can move the track in vitro using motility assays. In order to ascertain whether the N-terminal domain of MukB fulfils any of these activities we recombinantly expressed the first 342 residues of the protein for further biochemical characterisation.

2. Materials and methods

2.1. Construction of pETMuk342 and pETMukR250A

The N-terminal 342 residues of MukB were amplified using the polymerase chain reaction from the plasmid pAX814 [1] and cloned into *Nde*I/*Eco*RI cut pET17b (AMS Biotechnology Ltd). The resulting plasmid pETN-term342 was digested with *Eco*RI and ligated to oligonucleotides encoding the poly-His tag EFRGSHHHHHH followed by a stop codon. The authenticity of this construct, pETMuk342 was checked by sequencing and restriction digests.

2.2. Expression and purification of Muk342

Freshly transformed pLysS cells harbouring pETMuk342 were grown in 2×YT supplemented with ampicillin (100 μg/ml) and chloramphenicol (34 μg/ml) at 37°C until an absorbance at 600 nm of 1. The temperature was then reduced to 20°C and the expression of Muk342 induced by the addition of IPTG to a final concentration of 0.1 mM. The bacteria were allowed to grow for a further 4 h before harvesting by centrifugation. The cell pellets were frozen and stored in liquid nitrogen. Cell pellets were resuspended in Buffer A (50 mM potassium phosphate, pH 7.4, 5 mM β-mercaptoethanol, 5 mM magnesium acetate (MgOAc), 300 mM NaCl and 100 mM imidazole) supplemented with Complete EDTA Free Protease Inhibitor Cocktail tablets (Boehringer Mannheim) and incubated on ice with lysozyme (0.1 mg/ml), deoxyribonuclease I (40 μg/ml) and Triton X-100 (0.05%) for 20 min. The supernatant was clarified by centrifugation (27 000×*g*, 50 min, 4°C) and the cell pellet discarded. All steps were performed at 4°C using an FPLC system (Pharmacia Biotech). Muk342 was purified by passing the clarified lysate over a 4-ml Ni-NTA resin column (Qiagen) equilibrated in Buffer A. After extensive washing of the column in Buffer A, Muk342 was eluted from the column using 100 mM potassium phosphate, pH 4.5, 5 mM β-mercaptoethanol, 5 mM MgOAc, 100 mM NaCl. The pH of the eluant was rapidly adjusted to pH 6.5 and the protein loaded onto a 1-ml HiTrap SP column (Amersham Pharmacia Biotech) equilibrated in Buffer B (50 mM potassium phosphate, pH 6.5, 2 mM DTT, 5 mM MgOAc). Muk342 was eluted from the column using Buffer B supplemented with 400 mM NaCl. The peak fractions were concentrated using a Centricon 30 (Amicon) and the protein solution supplemented with 20% glycerol before storage in liquid nitrogen. The concentration of Muk342 was determined spectrophotometrically at a wavelength of 280 nm in 6 M guanidine hydrochloride using a calculated extinction coefficient of 18 200 M^{-1} cm^{-1}. All protein concentrations are expressed per Muk342 monomer and the authenticity of the purified protein was confirmed by N-terminal sequencing.

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2.3. Cloning and expression of GST-FtsZ

The full length FtsZ gene was amplified from genomic DNA prepared from BL21(DE3) cells, digested with *Mfe*I and cloned into the *Eco*RI site of pGEX-2T (Amersham Pharmacia Biotech) to form the plasmid pGEX-FtsZ. The authenticity of the construct was checked by sequencing and restriction digests. Overexpressed GST-FtsZ was purified from DH5α cells by passing the high speed supernatant from lysozyme lysed cells over a glutathione-Sepharose column (Amersham Pharmacia Biotech) equilibrated in lysis buffer (20 mM potassium phosphate, pH 7.4, 2 mM DTT, 5 mM MgOAc and 140 mM NaCl). After extensive washing with lysis buffer GST-FtsZ was eluted from the column using 50 mM Tris, pH 7.5, 2 mM DTT, 5 mM MgOAc, 100 mM NaCl and 20 mM glutathione. GST-FtsZ was concentrated using a Centricon 30 and passed over a PD-10 desalting column (Amersham Pharmacia Biotech) equilibrated in the elution buffer minus the glutathione before storage in liquid nitrogen. The concentration of the purified protein was determined at 280 nm using an extinction coefficient of 44 800 M^{-1} cm^{-1} and is expressed per GST-FtsZ monomer.

2.4. FtsZ pelleting assays

All pelleting assays were performed in 50 mM potassium phosphate, pH 6.5, 2 mM DTT, 5 mM MgOAc, 50 mM KCl and 2.5 mM GTP using 40-μl volumes and were immediately spun after mixing in a TLA100.1 rotor using a Beckman TLX ultracentrifuge at 50 000 rpm for 15 min at 20°C. Assays using a fixed concentration of Muk342 (2.0 μM) were titrated against increasing concentrations of GST-FtsZ (0.87–21 μM). After the spin supernatants were removed and added to 20 μl of SDS-gel loading buffer and the pellets resuspended in 60 μl SDS-gel loading buffer. Equal volumes of pellet and supernatant were analysed by SDS-PAGE and after electrophoresis the gels were stained with Coomassie Brilliant Blue R-250 and then thoroughly destained before analysis. In order to determine the relative amounts of protein in the supernatant and pellet fractions the gels were scanned using a Molecular Dynamics Model 300A densitometer and quantitated using the associated ImageQuant software. The measured dissociation constants and maximal binding were obtained by fitting the fraction of Muk342 pelleted vs. the amount of FtsZ pelleted to a rectangular hyperbola using Kaleidograph 3.0 (Synergy Software). Values were corrected for the small amounts of Muk342 (typically 5–10%) that pelleted in the absence of GST-FtsZ.

2.5. Measurement of GTP turnover by Muk342 and GST-FtsZ

All steady state ATPase and GTPase activities were measured using a pyruvate kinase/lactate dehydrogenase linked assay system as described in [3] except that the reactions were performed at 20°C in the presence of 2 mM Mg-GTP using FtsZ pelleting assay buffer. Assays were performed by first measuring the turnover of GTP by 1 μM GST-FtsZ. Muk342 (0.7 μM) was then added to the same assay mixture and the new combined rate of GTP hydrolysis recorded. In all the assays performed this new rate of GTP turnover was faster than the FtsZ alone rate. The rate of GTP turnover by Muk342 was measured separately under identical conditions. Assays comparing the rates of ATP and GTP turnover by Muk342 were also measured using this linked assay but were performed in 50 mM potassium phosphate, pH 7.4, 2 mM DTT, 5 mM MgOAc and 200 mM NaCl at 20°C and were carried out in duplicate.

3. Results

3.1. Expression and characterisation of Muk342

We expressed, using recombinant techniques, the N-terminal 342 residues of MukB fused to a C-terminal poly-histidine tag (Muk342) to facilitate the identification and rapid purification of the protein from bacterial lysates. Gel filtration chromatography of the purified Muk342 indicated a Stokes Radius (R_S) of 2.63 nm and an estimated molecular weight of 45.8 kDa (calculated polypeptide M_r = 39.7 kDa). The Stokes radius of Muk342 is very similar in value to that of the kinesin motor domain (residues 1–340, R_S = 2.90 nm, M_r = 37.8 kDa [4]) and an Ncd motor domain (residues 327–700, R_S = 2.95 nm, M_r = 42.3 kDa [4]) indicating that like these proteins the N-terminal domain of MukB is monomeric.

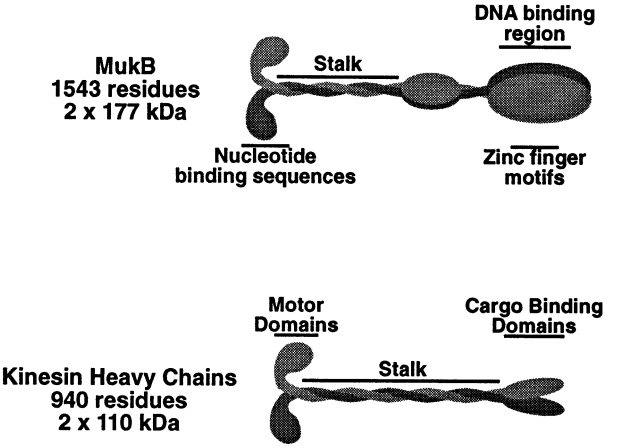

Fig. 1. Schematic comparison of MukB and the kinesin heavy chain. MukB forms homodimers and has a domain structure similar to that of the kinesin heavy chain. The twin N-terminal domains, of ~340 residues each, have nucleotide binding motifs and the larger C-terminal globular region (residues 665–1543) can be split into two domains of unequal size separated by a small region of predicted coiled coil [1].

Previous studies indicated that the full length MukB protein could be photoaffinity crosslinked to both ATP and GTP in the presence of Zn^{2+} ions although no ATPase or GTPase activity was detected [2]. However, we found that purified Muk342 is able to hydrolyse both ATP and GTP in the presence of Mg^{2+} ions. When Zn^{2+} ions replaced Mg^{2+} ions in the assay mixture (using a non-phosphate containing buffer) Muk342 was found to precipitate (data not shown). The rates of nucleotide hydrolysis indicate that Muk342 has a slow basal activity turning over ATP and GTP at rates of 1.23 min^{-1} (2.64 mM ATP) and 0.17 min^{-1} (2.64 mM GTP), respectively, and are within the same order of magnitude as those reported for kinesin [5]. The absence of NTPase activity in the previous study may have been due to a number of factors such as protein inactivation during the relatively long purification protocol or through the omission of cofactor ions [2].

3.2. MukB binds with high affinity to the bacterial tubulin homologue FtsZ

We overexpressed FtsZ in *E. coli*, fused to the C-terminus of glutathione *S*-transferase (GST-FtsZ) to allow its rapid purification from bacterial lysates. The purified GST-FtsZ fu-

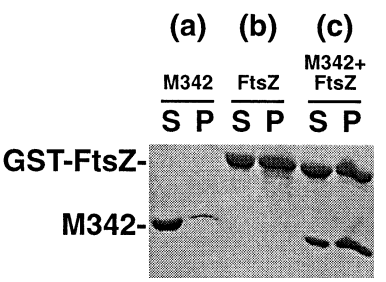

Fig. 2. Greyscale image of Coomassie-stained SDS-gel showing the pelleting of: a: Muk342 alone (3.75 μM); b: GST-FtsZ alone (M_r = 66 kDa, 7 μM); and c: Muk342 (3.75 μM) plus FtsZ (7 μM). All assays contained 2 mM GTP. S = supernatant, P = pellet.

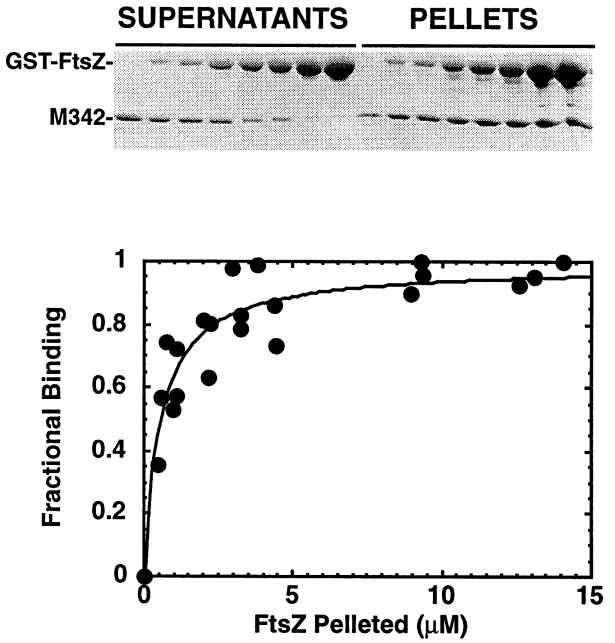

Fig. 3. Greyscale image of a typical Coomassie-stained SDS-gel showing the pelleting of a fixed concentration of Muk342 (2 μM) with increasing concentrations of GST-FtsZ (0.87–21 μM). Assays were performed in the presence of 2.5 mM GTP and the binding isotherm gave a K_d value of 0.55 ± 0.07 μM with a maximal binding value of 0.98.

sion protein shows an identical sedimentation behaviour to non-fusion FtsZ protein, with around 50–60% of the GST-FtsZ pelleting in the presence of 2 mM GTP [6] (Fig. 2b). Only this proportion of the GST-FtsZ remains polymerised due to instability of the polymers, presumably caused by the hydrolysis of bound GTP to GDP. Control experiments performed in the absence of GTP results in less than 5% of GST-FtsZ pelleting indicating that GTP is essential for GST-FtsZ polymerisation (data not shown) [7]. This is in many respects similar to the behaviour of tubulin which displays dynamic instability [8], however, inclusion of the drug taxol prevents depolymerisation of microtubules and allows their complete sedimentation. As yet a similar method to preferentially stabilise FtsZ polymers in the absence of GTP has not been found.

Pelleting of GST-FtsZ in the presence of Muk342 results in greater than 65% of the MukB co-sedimenting with the polymerised FtsZ clearly demonstrating a strong interaction between the two proteins (Fig. 2c). Under the assay conditions used less than 5% of Muk342 pellets in the absence of GST-FtsZ (Fig. 2a).

The interaction between Muk342 and GST-FtsZ was further characterised by pelleting a fixed amount of Muk342 with increasing amounts of GST-FtsZ in the presence of 2.5 mM GTP (Fig. 3). These assays clearly demonstrated the binding of FtsZ to a single site on Muk342 and the dissociation constant was estimated to be 0.55 (± 0.07) μM. Thus the interaction between Muk342 and FtsZ is relatively strong even in the presence of high concentrations of GTP. We found that the inclusion of either 4 mM AMPPNP or 4 mM ADP in the assays did not significantly alter the affinity of Muk342 for GST-FtsZ.

This absence of a nucleotide dependence in these assays may be due to a number of factors. Firstly, although we have demonstrated that Muk342 can hydrolyse both ATP and GTP we do not at present know which of these nucleotides is the natural substrate for the protein. In addition, the binding assays require the inclusion of relatively high concentrations of GTP and, if the affinity of Muk342 for ATP is significantly less than that for GTP it will not bind either ADP or AMPPNP under these conditions.

In order to investigate this latter possibility we measured the turnover of ATP and GTP by Muk342 at two different nucleotide concentrations. The rate of GTP turnover by Muk342 was found to be unaffected by lowering the GTP concentration two-fold (0.163 and 0.173 min^{-1} at 1.32 and 2.64 mM GTP, respectively) whereas the rate of ATP turnover decreased around two-fold at the lower ATP concentration (0.76 and 1.23 min^{-1} at 1.32 and 2.64 mM ATP, respectively). These results are consistent with Muk342 having a much higher affinity for GTP compared with ATP. As the rate of GTP hydrolysis appears to be saturated at around 1–2 mM GTP this would suggest a low micromolar affinity for this nucleotide compared with a millimolar affinity for ATP. This in contrast to kinesin which shows a high affinity for ATP compared with GTP [9]. These results suggest that the in vivo fuel for MukB is GTP and also helps to explain why the pelleting assays performed with FtsZ (plus 2.5 mM GTP) failed to show any nucleotide dependence in the presence of either ADP or AMPPNP. We also tried using GMPPNP instead of GTP in the assay buffer but found that this analogue did not support the polymerisation of GST-FtsZ.

3.3. Accelerated nucleotide turnover in the presence of FtsZ

We also looked for evidence that FtsZ can stimulate the turnover of nucleotide by Muk342. These assays employed a pyruvate kinase/lactate dehydrogenase linked assay which detects the production of GDP and measured the rate of GTP hydrolysis by Muk342 alone (0.06 ± 0.02 min^{-1}, $n = 3$), FtsZ alone (0.81 ± 0.09 min^{-1}, $n = 4$) and Muk342 plus FtsZ (1.06 ± 0.13 min^{-1}, $n = 4$) (Fig. 4). The latter combined rate of GTP hydrolysis was significantly greater than ($P < 0.05$) the sum of the Muk342 alone and FtsZ alone hydrolysis rates and suggests one of two possible explanations. Firstly, and characteristic of motor proteins, FtsZ stimulates the GTPase

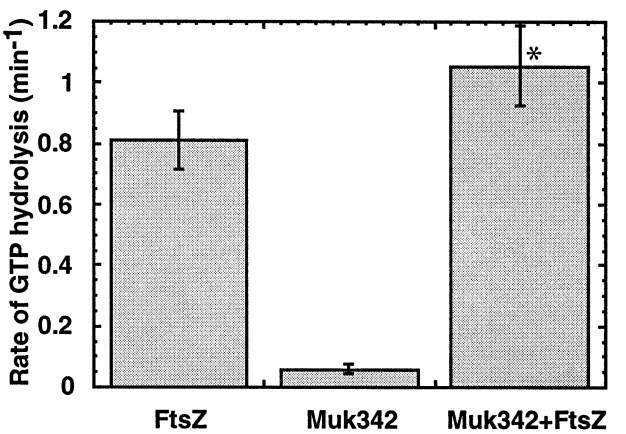

Fig. 4. Histogram showing the average GTP hydrolysis rates of GST-FtsZ alone, Muk342 alone and Muk342 plus GST-FtsZ. The latter combined rate of GTP turnover was significantly greater (*$P < 0.05$) than the sum of the GST-FtsZ alone and Muk342 alone rates.

activity of Muk342 around four-fold from 0.06 to 0.25 min^{-1}. Alternatively, and a possibility that we cannot at present formally exclude, is that Muk342 weakly stimulates ($\sim$1.3-fold) the GTPase activity of FtsZ. However, we believe this latter possibility is unlikely as any stimulation of FtsZ's GTPase activity would drive the protein into its GDP state more quickly in the presence of Muk342. This would tend to disassemble the FtsZ polymers as GDP does not support the polymerisation of FtsZ [6]. Analysis of the binding assays indicates that the addition of Muk324 to FtsZ does not significantly alter the amount of FtsZ sedimenting (data not shown) thus favouring the conclusion that FtsZ stimulates the GTPase activity of Muk342.

4. Discussion

Previous studies have demonstrated that the MukB protein is involved in chromosome partition in *E. coli* [1] and a number of reviews have suggested that, consistent with this role, the protein is a bacterial motor protein [10,11]. We have attempted in this study to address this central and important question by characterising the N-terminal domain of MukB.

4.1. Interaction of MukB with FtsZ

A growing number of studies have demonstrated that FtsZ can assemble into a range of filamentous structures that resemble those formed by the polymerisation of tubulin (reviewed in [12]). Taken together with the recent solving of the crystal structures of both proteins [13,14], which demonstrated a strong degree of structural homology, these studies suggest that FtsZ is a prokaryotic homologue of tubulin. However, unlike tubulin we are unfortunately limited in the types of biochemical experiments we can perform with FtsZ due to the absence of taxol like inhibitors of FtsZ depolymerisation and the necessity for including GTP in order to drive the polymerisation of FtsZ. Nevertheless we were able to perform a series of experiments that demonstrate that Muk342 binds with high affinity to polymerised FtsZ in the presence of GTP and that there is a single binding site on Muk342 for FtsZ.

The affinity of Muk342 for FtsZ ($K_d = 0.55$ μM) under these conditions appears to be much higher than that of monomeric kinesin for microtubules in the presence of ATP ($K_d = 9$ μM, [15]). This may suggest differences in the mechanochemical coupling of MukB and kinesin or that the two proteins have similar patterns of affinity in different nucleotide states which simply differ in their respective magnitudes. However, until we are able to stabilise FtsZ in the absence of GTP we will not be able to distinguish between these possibilities.

Attempts to measure the number of binding sites on FtsZ for Muk342 have so far been hampered due to Muk342 non-specifically binding to the FtsZ at high ratios of Muk342 to FtsZ (data not shown). This behaviour has been observed before with monomeric kinesin constructs under similar conditions of high motor concentrations to track [16].

The results of the GTPase assays indicated that either FtsZ stimulated Muk342's GTPase or that Muk342 was accelerating FtsZ's GTPase. We believe this latter possibility is unlikely as the available biochemical and structural evidence suggest that the polymerisation of FtsZ is very similar to that of tubulin, such that it is GTP driven and that GTP hydrolysis results in a dynamic assembly process. Any stimulation of FtsZ's GTPase activity would therefore be expected to shift the equilibrium towards disassembly due to the faster conversion of GTP to GDP, which does not support FtsZ assembly [6]. Analysis of the binding assays suggests that the inclusion of Muk342 does not significantly alter the amount of FtsZ pelleting supporting the conclusion that FtsZ stimulates the GTPase of Muk342.

Although the four-fold increase in Muk342's GTPase activity by FtsZ may appear rather modest in comparison to microtubule 1000-fold stimulation of kinesin's ATPase it is similar in magnitude to that reported for the microtubule stimulated ATPase of the kinesin related protein Kar3 [17]. For kinesin related motors the maximal rate of microtubule activated ATP hydrolysis appears to be related to the speed at which the motors translocate microtubules, such that a fast moving motor such as kinesin has a high ATPase rate relative to a slower moving motor such as Kar3 [18]. However, because we had to achieve a balance between swamping the signal from the Muk342 GTPase with the much faster GTPase activity from FtsZ we were unable to use conditions under which the Muk342 would be saturated by FtsZ. The maximal rate of GTP turnover by Muk342 may well therefore be faster at higher saturating FtsZ concentrations.

We have made some preliminary attempts to visualise the movement of FtsZ by Muk342 using a green fluorescent protein fusion (GFP) of FtsZ (FtsZ-GFP). However, visualisation of the polymerised FtsZ-GFP indicates that it forms extremely long filaments which aggregate on the glass surface and form aster like structures which are unsuitable substrates for motility assays. The use of FtsZ in motility assays will require further biochemical developments that allow the formation of stable, shorter polymers.

4.2. Mechanisms of chromosome partition

Chromosome partition requires that the bacterial nucleoids each move apart by around half a cell length (500 nm) and these findings raise the exciting possibility that *E. coli* possesses a rudimentary mitotic apparatus. Electron microscopy of MukB estimated its length to be around 50–60 nm and demonstrated that it can adopt at least two conformations: straight rods and a 'folded' V-shape [2]. The transition between these two conformations could, in principle, result in a small movement of the nucleoids, however, it would clearly be of insufficient magnitude on its own to separate the nucleoids to their correct positions. A switch between the two conformations may instead represent a regulatory control mechanism in the same way as both myosin II [19] and kinesin [20] are found, under physiological conditions, in inhibited folded conformations.

Movement of the nucleoids could in principle result from their translocation along two sets of oppositely oriented polar FtsZ filaments running from the cell equator to the quarter and three quarter cell lengths. Is there any evidence at present that *E. coli* contains such cytoskeletal elements? Immunoelectron microscopy studies first indicated that FtsZ concentrates at the cell equator where it forms a circumferential ring known as the Z ring [21]. Later studies with GFP-tagged FtsZ, which allowed a dynamic visualisation of the protein, also demonstrated its localisation to the Z-ring [22]. However, a significant proportion of the cells appeared to contain two juxtaposed rings, which due to the spatial resolution of the technique, it is unclear whether they are in fact formed from

two separate FtsZ rings or short FtsZ spiral structures. Interestingly the double ring pattern was associated with a larger internucleoid space than when just a single ring was present and may indicate a change in the polymerised form of FtsZ which allows it to serve as a track for MukB during chromosome partitioning.

4.3. Evidence that MukB is a bacterial linear motor protein

The data presented here for MukB are consistent with those expected of a motor protein and with its proposed role in the movement of bacterial chromosomes during partition. Firstly, motor proteins require that energy is expended in the form of nucleotide hydrolysis and we have demonstrated that MukB can hydrolyse nucleotides to provide the fuel for force generation. Secondly, motors require track proteins to walk along or generate force on and we have strong evidence that MukB can bind to proteins that can polymerise to form such tracks. Thirdly, FtsZ is able to accelerate the turnover of nucleotide by Muk342, which is indicative of a coupling between a motor's nucleotide hydrolysis cycle and a motor's mechanical force generating cycle. Obviously as a final coup de grâce we would like to be able to demonstrate the movement of FtsZ filaments by MukB using an in vitro motility assay and we are at present working towards this goal.

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