Title: FK506-binding protein FklB is involved in biofilm formation through its peptidyl-prolyl isomerase activity

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Abstract

FklB is a member of the FK506-binding proteins (FKBPs), a family that consists of five genes in *Escherichia coli*. Little is known about the physiological and functional role of FklB in bacterial movement. In the present study, FklB knock-out mutant $\Delta fklB$ presented an increased swarming and swimming motility and biofilm formation phenotype, suggesting that FklB is a negative regulator of these cellular processes. Complementation with Peptidyl-prolyl isomerase (PPlase)-deficient *fklB* gene (*Y181A*) revealed that the defects in biofilm formation were not restored by *Y181A*, indicating that PPlase activity of FklB is modulating biofilm formation in *E. coli*. The mean cell length of $\Delta fklB$ swarming cells was significantly smaller as compared to the wild-type BW25113. Furthermore, the mean cell length of swarming and swimming wild-type and $\Delta fklB$ cells overexpressing *fklB* or *Y181A* was considerably larger, suggesting that PPlase activity of FklB plays a role in cell elongation and/or cell division. A multi-copy suppression assay demonstrated that defects in motility and biofilm phenotype were compensated by overexpressing sets of PPlase-encoding genes. Taken together, our data represent the first report demonstrating the involvement of FklB in cellular functions of *E. coli*.

Introduction

The previously prevalent view of bacteria development was considered to be the planktonic form of life. That is, unicellular organisms that grow as individual entities. This view has changed as it has been found that bacteria, under given conditions, behave as multicellular groups that grow on nutrient-rich surfaces, secrete a polysaccharide material, through a process called swarming motility. Swarming motility is mainly driven by rotating flagella, and swarming bacteria generally appear to be elongated as a result of cell division suppression [1][2]. In contrast to swarming, swimming describes a mode in which cells move within aqueous environments, not in groups, but independently, by operating their rotating flagella [3]. A comparable type of multicellular behavior is the biofilm formation where bacteria form sessile communities and disperse by secreting proteins and surfactants extracellularly [4]. Biofilms are complex systems and can be composed of multiple species [5]. Environmental conditions and coordinated life cycles can affect or set off heterogeneity and include, among other, expression of genes and proteins, as well as post-translational protein modifications (PTMs), that could alter environmental sensing and signal transduction [6][7]. Various PTMs such as glycosylation, N-terminal modifications and phosphorylation are few of the

functional properties of Peptidyl-prolyl *cis/trans* isomerases (PPlases). PPlases, being ubiquitous among all organisms, are key regulators of numerous highly important biological processes; they accelerate the rate of *in vitro* protein folding and they have the ability to bind proteins and act as chaperones.

Additionally, PPlases catalyze the folding of newly synthesized protein targets, particularly those that have peptide bonds in the *trans* conformation. They are also able to alter the structure and conformation of mature proteins thus affecting their intermolecular interactions [8]. In bacteria and other organisms, there are three characterized PPlase subfamilies; the Cyclophilins, the FK506-binding proteins (FKBPs), and the Parvulins. *E. coli* FKBP family consists of five genes; *fkpA*, *fkpB*, *fklB*, *slyD* and *tig*, none of which is essential for growth [9]. FKBPs are found to be involved in a diverse series of cellular processes such as cell division [10], stress response regulation and development [11], gene regulation through transcription and translation [12], and most importantly, virulence and pathogenicity [13].

E. coli FklB (or FKPB22) possesses PPlase activity, exists in solution as a homodimer and shares a significant homology with the protein Mip (macrophage infectivity potentiator) that is identified in a number of human pathogenic bacteria, such as *Legionella pneumophila*, *Neisseria gonorrhoeae*, and *Chlamydia trachomatis* in the psychrotrophic bacterium *Shewanella* sp. SIB1 [14] but also in the plant pathogen *Xanthomonas campestris* [15]. *Shewanella* SIB1 FKBP22 is composed of two monomers that are connected at their N-termini, bearing a V-shaped structure. Within the monomer, a 40-residue long α -helix separates the N- and C-terminal domain [16]. An almost identical tertiary structure appears to be assumed by the *E. coli* FklB [17]. Data suggest that the probable PPlase binding site of SIB1 FKBP22 for a protein substrate is located at its C-terminal domain. Abrogation of SIB1 FKBP22 PPlase activity did not significantly affect its chaperone function [18].

This paper describes a new approach to investigate the physiological role and assess the PPlase and chaperone function of FklB through a series of phenotypic methods. We focused on genetic and biochemical approaches to assess the swarming and swimming motility, biofilm and cell length phenotypes in *E. coli*, caused either by the loss of *fklB* or by the overexpression of *fklB* and of PPlase-deficient *fklB* (*Y181A*) gene. We found that deletion of *fklB* resulted in an enhancement of motility and biofilm, as well as a decrease of swarming cells' length. Complementation with *fklB* gene, in the mutant strain Δ FklB, suppressed the *fklB* deletion motility and biofilm phenotype, while overexpression of *Y181A* suppressed only the motility phenotype. Overexpression of *fklB* or *Y181A* gene exhibited opposite effects on the mean cell length of swarming and swimming cells. We also used a multi-copy suppression approach to assess if overexpression of other PPlase-encoding genes may suppress the Δ *fklB* strain motility and biofilm phenotypes.

Results & Discussion

PPlase and chaperone activity of FkIB

Initially, we examined the PPlase activity of FkIB with a standard PPlase assay of isomer-specific proteolysis by chymotrypsin, described by Kofron [19]. Based on the protein alignment of *E. coli* FkIB (Ec_4207) with the fully characterized human FkpB12 (hfkpB12), we located the FkIB's putative active sites. We then constructed an active site mutated form of FkIB, Y181A, which we used in the PPlase assay in comparison to the wild-type FkIB. N-succinyl-Ala-Ala-Pro-Phe-pnitroanilide was used as a substrate known to mimic the internal peptidyl-prolyl moiety of proteins containing proline.

We found that Y181A had no measurable isomerase function, whereas the catalytic efficiency of FkIB was 1.50 ± 0.0013 (Kcat/Km), suggesting that substitution of Y181 had a significant effect on its activity (Fig. 1A). This result suggests that Y181A, located on its C-terminal domain, is involved in the catalytic function of the enzyme. Previous findings have demonstrated that the catalytic efficiencies of other mutant forms of FkIB indicate that W157 and F197 are also critically important for the isomerase activity of SIB1 FKBP22 [20].

Next, we sought to determine the chaperone activity of FkIB by measuring its ability to suppress the thermal aggregation of citrate synthase (CS) [21], while also evaluating any effects on it, caused by the induced active site mutation. CS tends to aggregate at high temperatures because of hydrophobic interactions between unfolding intermediates and results in the formation of high molecular weight particles. Proteins with a chaperone function are able to recognize and bind to these unfolding intermediates and therefore keep their concentration low in solution. We observed that wild-type FkIB presents a chaperone function, whereas the inhibition of the CS aggregation from Y181A appears to increase in proportion to its concentration (Fig. 1B).

However, we noticed an increased ability of Y181A to inhibit the formation of CS aggregates, in comparison to the wild-type FkIB. This may be an indication that the loss of FkIB's PPlase activity improves its function as a chaperone.

It has been previously shown that the PPlase and chaperone activity of SIB1 FKBP22 reside in two structurally unrelated domains, but not necessarily functionally independent domains. Mutations at its PPlase active site do not critically affect its chaperone function, an indication that SIB1 FKBP22 does not require PPlase activity for protein folding. However, the authors highlight the importance and requirement of the chaperone domain for the PPlase activity, as a way of enabling the formation of folding intermediates [20]. Several other studies have also indicated that the presence of chaperone activity improves PPlase activity [22] [23]. The substrates are bound to the chaperone site and are subsequently transferred to the PPlase site, where the peptidyl-prolyl bonds of the proteins are being isomerized. Protein molecules, that exited the PPlase site with an incorrect peptide-prolyl bond, are re-attached to the chaperone region and the procedure is repeated [23]. Therefore, it could be assumed that the chaperone activity of FkIB may offset a high PPlase activity and concurrently the loss of PPlase activity might allow structural changes

that increase the chaperone activity. Elucidating the structural relationship and association of the two functions is very important in order to uncover the role of the PPIase family in major cellular processes.

Role of FklB in swarming and swimming motility

The role of FklB was examined under swarming and swimming conditions, by inoculating the center of swarming (LB-glucose, 0.5% agar) and swimming plates (LB, 0.3% agar) with liquid cultures expressing and/or lacking the *fklB* gene (Fig. 2). We found that the mutant Δ FklB strain formed considerably larger swarming and swimming colonies in comparison to the control strain, BW25113, indicating that the loss of FklB is responsible for the observed phenotype (Fig. 2A-B). In order to validate that FklB functions as a swarming and swimming motility repressor we examined the phenotypes of the Δ FklB strain and of the control strain overexpressing the *fklB* gene (strain BW25113(FklB)). We found that the Δ FklB(FklB) reverted the hyper-swearer or hyper-swimmer phenotype to wild-type, while BW25113(FklB) further suppressed the phenotype beyond the wild-type levels (Fig. 2A-D). This observation supports our initial hypothesis that the lack of FklB was the causative factor of the increased swarming and swimming motility. Growth rates of Δ FklB or BW25113 (FklB) strain liquid cultures were comparable to the control's, suggesting that the increased motility phenotype was not attributed to an increased growth.

Subsequently, we checked the involvement of the PPIase activity of FklB protein in swarming and swimming motility by following the same conditions. Strain Δ FklB(Y181A) did not seem to differ from the corresponding strain Δ fklB(FklB), even in highest IPTG concentrations, as both displayed no motility on swarm or swim plates, suggesting that the PPIase activity of FklB is not likely to be involved in the mechanism. Overall, we found that the expression of FklB and its mutant form, Y181A, is able to restore the wild-type phenotype at all IPTG concentrations (0.1-0.5 mM). This confirms that the presence of FklB is indispensable for maintaining a normal phenotype, perhaps through pre- and post-translational modifications or indirect target protein interactions that control a wide range of cellular processes, including motility [24] [25]. The negative regulation of swarming and swimming motility in *E. coli* by certain PPIase family members was previously shown [26][27]. FkpB proteins are found to be involved in bacterial motility, for example, an increase in the transcript levels of *fklB* gene was observed in *P. mirabilis* swarming cells [28]. Another example is the GldI protein of the microorganism *F. johnsoniae*, a lipoprotein homologous to FKBP, essential for gliding mobility [29]. Although the structure of almost all FKBP proteins has been extensively studied, our knowledge about their biological role still remains limited. We already know that FKBP catalyze the refolding of peptides preceding proline at polypeptide chains, as well as that all exhibit some PPIase activity, but there are still several unanswered questions about their physiological role.

FklB is suppressing *E. coli*'s biofilm formation ability

Swarming motility and biofilm formation relationship seems to be complex and although both conditions share some common constituents, they greatly differ. Specifically, the use of flagella is necessary for

biofilm initiation, but motility is also required for its initiation, as well as dispersion and release of bacteria [30]. However, it is not clear whether there is an inverse regulation of swarming motility and biofilm formation, as conflicting data have been published. For example, an increased EPS production suppressed swarming motility, but enhanced biofilm formation among laboratory isolates [31]. Biofilm formation, as well as swarming and swimming motility, was suppressed by overexpressing the cyclophilin PpiB. However, this involvement of PpiB in the biofilm formation phenotype does not involve its prolyl isomerase activity [32]. Biofilm formation was explored for FkIB in order to elucidate the role of its PPIase function in this multicellular behavior, but also to investigate into its relation to swarming. To this means, we initially compared the biofilm formed by the control BW25113 and the mutant strain Δ FkIB and we noticed that the Δ FkIB strain was capable of a greatly increased biofilm formation (Fig. 3). We hypothesized that the increased biofilm formation by Δ FkIB was attributed to the absence of the FkIB protein and in order to clarify this we tested the biofilm formation under the same conditions of the strain Δ FkIB(FkIB) as well as the strain BW25113(FkIB). Indeed, we noticed the restoration of the wild-type phenotype, when FkIB was expressed at intermediate IPTG concentrations (0.1-0.25 mM), in the mutant strain (Δ FkIB(FkIB)) and in the wild-type strain (BW25113(FkIB)). Interestingly, we further detected a biofilm repression phenotype when FkIB was overexpressed (0.5 mM IPTG), either in BW25113(FkIB) or in Δ FkIB(FkIB) strain, suggesting that FkIB bears a key role in biofilm formation (Fig. 3A-B).

Similarly, we tested the strains that overexpress the mutant protein Y181A, Δ FkIB(Y181A) and BW25113(Y181A). The results showed that the biofilm of strain Δ FkIB(Y181A), did not differ from the mutant strain Δ FkIB, even in the presence of high levels of IPTG (0.25 and 0.5 mM). The overexpression of the mutant Y181A did not cause a restoration of the wild-type phenotype. Based on these results, we can conclude that FkIB's PPIase activity is involved in this multicellular behavior (Fig. 3A).

Additionally, the strain BW25113(Y181A), in the absence or presence of low levels of IPTG (0.1 mM), showed similar biofilm formation ability to the control BW25113. However, we noticed that even though the strain BW25113(Y181A) at 0.25 and 0.5 mM IPTG, showed an important decrease in biofilm formation, that decrease was slightly lower than BW25113 (FkIB) ((Fig. 3B). These observations seem to suggest that FkIB has an important role in suppressing the biofilm formation phenotype of *E. coli* and that its PPIase activity is indispensable for this involvement.

PPIase family members can functionally replace FkIB in swarming, swimming and biofilm cells

Previous research has demonstrated that two members of the PPIase family are functionally linked in yeast cells. It was found that although these proteins do not bind or catalyze the same peptides, they can generate conformational changes to substrates [33]. Another study has showed that a parvulin and a FKBP protein catalyze the *cis/trans* isomerization of peptide bonds in proteins with great homology [34]. Based on the above studies, we questioned whether the previously observed phenotypes of Δ FkIB mutant strain could reverse upon expression of members of the PPIase family. To this end, we separately introduced

and expressed plasmids that contained each gene belonging to the PPlase family; *fkpA*, *slyD*, *fkpB*, *tig*, *ppiA*, *ppiB*, *surA*, *ppiC* and *ppiD* into the Δ FkIB mutant strain and we compared the ability of each one of swarming, swimming and biofilm formation (Fig. 4).

Interestingly, we found that the multiple copies of all gene members of the PPlase family (0.5 mM IPTG), but not the member *tig* of the FKBP family, could rescue the hyper-swarming phenotype of the Δ FkIB mutant (Fig 4A). The hyper-swarming phenotype of Δ FkIB was restored to wild-type levels even at single copies of genes *fkpA*, *ppiD*, and of course *fklB* (0 mM IPTG). This observation could be evidential of a functional overlap between FKBP and parvulins, in swarming bacteria, perhaps hinting at the *cis/trans* isomerization of some common substrates.

The hyper-swimmer phenotype of Δ FkIB was rescued upon expression of the majority of PPlases, excluding the cyclophilins *ppiA* and *ppiB*. Every member of the FKBP family was able to complement FkIB's function in swimming cells even in single copies (0 mM IPTG). There was a significant increase detected at high expression levels of *tig* (0.5 mM IPTG), which indicates a unique involvement of the trigger factor protein in swimming motility (Fig. 4B).

Lastly, we checked the biofilm formation phenotype of the Δ FkIB could be abrogated by members of the PPlase family. We found that the expression of a great number of PPlases was not able to functionally replace FkIB. The members PpiA, PpiB, FkpB, Tig, PpiC, and PpiD did not rescue the increased biofilm of the mutant strain Δ FkIB, at low levels of expression (0 mM and 0.1 mM IPTG). Wild-type biofilm levels were recovered in the high-copy presence of PpiB, PpiC, and SurA (0.5 mM IPTG) and in the intermediate-copy presence of FkIB and FkpB (0.25 mM IPTG). Interestingly, we identified a biofilm suppression phenotype after expressing high-levels of FKBP encoding genes, *fkpA*, *fkpB*, *slyD*, and *fklB* (0.5 mM IPTG) (Fig. 4C).

The evidence from the above experiments point towards the idea that members of the PPlase family can compensate for the absence of FkIB, in swarmer, swimmer and biofilm *E. coli* cells. This functional replacement is even possible at very low copy numbers of PPlases, suggesting that there might be a substrate regulation pathway shared within the PPlase family. The data also indicate that there is a stronger physiological function commonality among members of the FKBP family through the regulation of cellular processes, post-translationally [26].

FkIB expression causes cell morphology alterations

Swarmer cells are described as elongated and hyperflagellated cells, that are able to migrate towards the edge of a swarming plate or a nutrient-rich surface, away from the initial colony [35] [36] [37]. We have previously examined *E. coli* cells in a planktonic phase that lack or overexpress the cyclophilin PpiB and found that in both cases the present an impaired cell division [38].

In this study, we examined the cell morphology of the control BW25113 and of the mutant Δ FkIB, as well as of the strains that overexpress FkIB; BW25113(FkIB), BW25113(Y181A), Δ FkIB(FkIB) and

$\Delta FkIB(Y181A)$, during swarming and swimming motility. The expression of plasmids that carried the *fklB* gene and its mutant, *Y181A*, was performed in the presence of 0.1, 0.25, and 0.5 mM IPTG. We microscopically observed all the above strains after Gram staining and after DAPI staining, using an optical and a fluorescence microscope, respectively, showing the expression of FkIB and Y181A at 0.25 mM IPTG (Fig. 5, 6).

In swarming cells, we observed that the absence of the *fklB* gene (strain $\Delta FkIB$) did not result in a differentiated cell phenotype when compared to the control strain (Fig. 5A). However, overexpression of the *fklB* gene, in both the wild-type and mutant strains, BW25113(FkIB) and $\Delta FkIB(FkIB)$, resulted into a phenotype characterized by elongated cells that have stopped dividing (Fig. 5A, 6A).

Additionally, a mixed population of normal size cells and cells that were not dividing was noted in the swarmer cells of strains BW25113(FkIB) and $\Delta FkIB(FkIB)$ overexpressing the *fklB* gene (Fig. 5A, 6A). For these cells, we noticed an abnormality in the septa formation, that did not allow the separation of the cellular membrane for cell division.

Regarding the swimming motility, we noted that the phenotype of the mutant strain $\Delta FkIB$ did not differ profoundly from the wild-type strain, BW25113. However, overexpression of the *fklB* gene in both strains, BW25113(FkIB) and $\Delta FkIB(FkIB)$, caused a pronounced cell elongation, in which it appeared that the cell division had been inhibited. Figure 5 shows that the cells of strain $\Delta FkIB(FkIB)$ formed multiple nucleoids, therefore we concluded that the replication of the genetic material was being done normally, while the cell wall and plasma membrane separation were not permitted (Fig. 5B, 6B).

The phenotype in both swarming and swimming cells seemed to reverse after the expression of the mutant gene, *Y181A*. Strains BW25113(Y181A) and $\Delta FkIB(Y181A)$ had a normal cell appearance under swarming and swimming conditions, that phenotypically corresponded to the wild-type strain. This observation led us to the conclusion that the cellular elongation of the strains BW25113(FkIB) and $\Delta FkIB(FkIB)$ was due to the increased levels of the FkIB protein. Summing up the results, it was concluded that the accumulation of the FkIB protein, in the swarmer and swimmer cells, caused phenotype alterations that were specific to the increased PPLase activity (Fig. 5, 6).

Materials and Methods

E. coli strains, growth conditions and growth rate assay

The bacterial strains and plasmids that were used in this study are described in Table 1. *E. coli* K-12 BW25113 and single-gene knockout mutants [39] were obtained from the *E. coli* Genetic Stock Center. Plasmid pCA24N, as well as plasmids pCA24N containing the PPIase encoding genes were obtained from the ASKA library of the NARA Institute [40]. Unless stated otherwise, bacteria were cultivated routinely in LB (Luria–Bertani) agar or broth at 37°C with aeration. When necessary, media were supplemented with chloramphenicol (25 µg/ml) or kanamycin (25 µg/ml) or ampicillin (100 µg/ml). The specific growth rates of the *E. coli* wild-type and mutant strains were determined by measuring the turbidity at 600 nm and C.F.U/ml for two independent cultures of each strain as a function of time with turbidity values less than 0.9.

Plasmids

The coding sequence of *EcFkIB* (NC_000913.3) was amplified using PCR and *E. coli* genomic DNA as a template. The primers used are *Ec.b4207.H.F*: 5'- CCAGGATCCGACCACCCCAACTTTTGACACC -3' and *Ec.b3349.H.R*: 5'- CGCAAGCTTTTAGAGGATTTCAGCAGTTC -3'. The fragment excised from amplified *EcFkIB* sequence was cloned between BamHI and HindIII sites of pPROEX-HTa, resulting in pPROEX-HTa *FkIB*. Y181A point mutation in *FkIB* was engineered using the gene-SOE method described by Horton [41]. The mutagenic primers used are *Ec.b4207.Y181A.F*: 5'- CCGCAGGAAGTGGCAGCTGGCGAGCGCGGCGCA-3' and *Ec.b4207.Y181A.R*: 5'- TGCGCCGCGCTCGCCATATGCCAGTTCCTGCGG-3'. The primary PCR products were purified and then used as templates for the second round of PCR. The final product was introduced into pPROEX-HTa, resulting in pPROEX-HTa Y181A. The nucleotide sequence of the gene encoding the mutant protein was confirmed by Sanger sequencing.

Heterologous expression of *FkIB* in *E. coli* and purification of recombinant protein

E. coli BL21(DE3) [F⁻ ompT gal dcm lon hsdS_B(r_B⁻ m_B⁻) λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5]) (Novagen, Madison, WI, USA) was used as a host strain for overproduction of a His-tagged form of *FkIB* or Y181A. Synthesis of recombinant proteins in *E. coli* BL21 (DE3) cells was initiated by addition of 0.25 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG) when the culture reached OD₆₀₀ of 0.6 and continued cultivation for additional 4h at 30°C. Recombinant proteins were purified with Ni-NTA chromatography (Ni²⁺-nitrilotriacetate, Qiagen) according to the manufacturer's instructions. To remove any imidazole and salts in the collected fractions, fractions were pooled accordingly and dialyzed against 35 mM Hepes buffer pH8.0 and 70 mM NaCl, for 12 h. Production levels and purity of the recombinant proteins were analyzed by 15% SDS-PAGE electrophoresis.

Motility assays

Overnight cultures of the different strains were grown, standardized to an OD600 of 1.2, and 3 µl used to stab or spotted at the center of swimming and swarming plates, respectively. The swimming plates were prepared with 0.3% Fluka agar, 1% Bacto-tryptone, 0.5% Yeast Extract and 1% NaCl. The swarming motility plates were prepared with 0.5% Fluka agar, 1% Bacto-tryptone, 0.5% Yeast Extract, 1% NaCl and 0.5% glucose. When necessary, media were supplemented with chloramphenicol (25 µg/ml) or kanamycin (25 µg/ml) and the appropriate amounts of IPTG. The plates were dried for 1-2 h at room temperature before being inoculated and were scanned after 20 h incubation at 30 °C. Petri dishes were scanned and the swarming and swimming areas were measured with the imaging software ImageJ. The experiments were carried out in three replicates.

Biofilm formation assay

The crystal violet biofilm assays were performed as previously described [4]. Briefly, BW25113 and the *fkIB* mutant strains containing either pPROEX-HTa FkIB or pPROEX-HTa Y181A were grown overnight in LB medium. The overnight cultures were 1:10 diluted in 100 µl of LB medium supplemented, when necessary, with appropriate concentrations of antibiotics and IPTG, and the biofilm was formed in covered 96-well microtiter dish for 20 h without shaking at 30°C. The cell suspensions were removed and turbidity was measured at OD600. The plates were washed once with sterile distilled H₂O to remove unbound bacteria and stained with 200 µl crystal violet (0.1% solution) for 20 min. Quantification was conducted by suspending the crystal violet stained cells in 200 µl of 20% acetone (in ethanol). Total biofilm formation was normalized by cell growth (turbidity at 600 nm) to avoid overestimating changes due to growth effects. As controls, BW25113 *fkIB* mutants with empty or pPROEX-HTa or pCA24N were used.

Peptidyl-prolyl *cis/trans* isomerase enzymatic assay

PPlase activity was tested using a chymotrypsin-coupled PPlase assay [19]. In this assay we measured the ability of FkIB or Y181A to convert the *cis* isomer of the synthetic oligopeptide substrate *N*-Suc-Ala-Leu-Pro-Phe-*p*-nitroanilide into the *trans* form. The assay reaction contained 50 mM Hepes buffer pH 8.0 and 100 mM NaCl, 50 µg α-chymotrypsin (dissolved in 1 mM HCl) (Fluka), 25 µM Suc-AAPF-pNA (5 mM stock dissolved in trifluoroethanol supplemented with 0.45 M LiCl) and the appropriate amount of recombinant FkIB or Y181A. The reaction was monitored at 4°C by the increase in absorbance at 390 nm (corresponding to the release of *p*-nitroanilide) using a HITACHI U-2800 spectrophotometer.

Citrate synthase thermal aggregation assay

Thermal denaturation of citrate synthase (0.25 µM final concentration, Sigma) was achieved by incubation at 45°C, in 40 mM Hepes pH: 7.5, for 15-20 min, in the absence or in the presence of additional proteins,

as previously described [21]. Aggregation of citrate synthase was measured by monitoring the increase in turbidity at 500 nm in a HITACHI U-2800 spectrophotometer equipped with a thermostatic cell holder. The absorbance change recorded is due to the increase in light scattering upon aggregation of citrate synthase. Protein disulfide isomerase (Sigma) was used in positive control reactions and albumin (Research Organics) was used in negative control reactions.

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Figures and figure legends

Figure 1. Prolyl isomerase and chaperone activity is lost in mutant Y181A compared to the wild-type protein FkIB.

A. PPlase activity of 0.25 μ M FkIB and Y181A. Mean values were obtained from three independent replicates and error bars represent standard errors.

B. Thermal aggregation of citrate synthase in the absence (●) and presence of (■) 0.25 μ M FkIB or 0.25 μ M Y181A (▲). The results are representative of three series of measurements carried out with different preparations of enzymes.

Figure 2. Prolyl isomerase activity of FliB causes a suppression of the swarming and swimming phenotype in *E. coli*. Swarming (A) and swimming (C) area of the ΔFliB mutant strain that overexpresses FliB and Y181A and swarming (B) and swimming area (D) of BW25113 that overexpresses FliB and Y181A, compared to the wild-type BW25113. Mean values were obtained from four independent replicates, and error bars represent standard errors. Statistical comparisons were made using ANOVA followed by Dunnett's multiple-comparison test. Asterisks indicate statistically significant differences ($P < 0.05$).

Figure 3. Prolyl isomerase activity of FkIB causes a suppression of the biofilm phenotype in *E. coli*. Biofilm formation of the $\Delta FkIB$ mutant strain that overexpresses FkIB and Y181A (A) and of the BW25113 that overexpresses FkIB and Y181A (B), compared to the wild-type BW25113. Mean values were obtained from four independent replicates, and error bars represent standard errors. Statistical comparisons were made using ANOVA followed by Dunnett's multiple-comparison test. Asterisks indicate statistically significant differences ($P < 0.05$).

Figure 5. Overexpression of FliB, but not Y181A, in Δ FliB causes a cell elongation phenotype in swarming and swimming *E. coli*. Δ FliB and Δ FliB overexpressing FliB or Y181A in pCA24N vector taken from swarming (A) and swimming (B) cells were examined after DAPI (upper row) or Gram (bottom row) staining by light and fluorescent microscopy and compared to the control, BW25113. Bars represent 10 μ m.

Figure 6. Overexpression of FkIB, but not Y181A, in BW25113 causes a cell elongation phenotype in swarming and swimming *E. coli*. BW25113 overexpressing FkIB or Y181A in pCA24N vector taken from swarming (A) and swimming (B) cells were examined after DAPI (upper row) or Gram (bottom row) staining by light and fluorescent microscopy and compared to the control, BW25113. Bars represent 10 μ m.