

Mechanistic Insights into MinD Regulation and Pattern Formation in *Bacillus subtilis*

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Abstract

Bacteria precisely regulate the place and timing of their cell division. One of the best-understood systems for division site selection is the Min system in *Escherichia coli*. In *E. coli*, the Min system displays remarkable pole-to-pole oscillation, creating a time-averaged minimum at the cell's geometric center, which marks the future division site. Interestingly, the Gram-positive model species *Bacillus subtilis* also encodes homologous proteins: the cell division inhibitor MinC and the Walker-ATPase MinD. However, *B. subtilis* lacks the activating protein MinE, which is essential for Min dynamics in *E. coli*. We have shown before that the *B. subtilis* Min system is highly dynamic and quickly relocates to active sites of division. This raised questions about how Min protein dynamics are regulated on a molecular level in *B. subtilis*. Here, we show with a combination of *in vitro* experiments and *in vivo* single-molecule imaging that the ATPase activity of *B. subtilis* MinD is activated solely by membrane binding. Additionally, both monomeric and dimeric MinD bind to the membrane, and binding of ATP to MinD is a prerequisite for fast membrane detachment. Single-molecule localization microscopy data confirm membrane binding of monomeric MinD variants. However, only wild type MinD enriches at cell poles and sites of ongoing division, likely due to interaction with MinJ. Monomeric MinD variants and locked dimers remain distributed along the membrane and lack the characteristic pattern formation. Single-molecule tracking data further support that MinD has a freely diffusive population, which is increased in the monomeric variants and a membrane binding defective mutant. Thus, MinD dynamics in *B. subtilis* do not require any unknown protein component and can be fully explained by MinD's binding and unbinding kinetics with the membrane. The generation of MinD patterns relies on the short-lived temporal residence of MinD dimers at the membrane.

eLife assessment

This **useful** study provides data suggesting that subcellular localization of the spatial regulator of cell division, MinD, is an intrinsic feature of the protein's ability to associate with the membrane as both a dimer and a monomer. These findings distinguish the behavior of MinD in *B. subtilis* from its counterpart in *E. coli* and suggest that there is not a need to invoke additional localization factors. However, all three reviewers agreed that the study is **incomplete**: experimentally, quantitation and assessment of MinD behavior in the presence of proteins previously implicated in its localization are missing, among other assays, and the molecular modeling necessary to support the authors' conclusion that their data support a reaction-diffusion model is completely absent. Finally, the manuscript itself is difficult to read with an overly long discussion and disorganized introduction and results sections, and it will require significant revision.

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Introduction

All cells face two fundamental challenges during their cycle: controlling the number of divisions and ensuring these divisions occur at the correct site. While these processes are extensively-studied in eukaryotes (1, 2), there remains significant uncertainty in bacterial cells. Most bacteria have simple morphologies, such as cocci or rod-shaped cells, and divide by binary fission. This division is governed by a complex protein machinery known as the divisome (3-5). The divisome's spatial organization is directed by the dynamic arrangement of a bacterial tubulin homolog, FtsZ (6, 7). FtsZ forms treadmilling protofilaments that condense at the division site (8-10), recruiting several cell wall synthesis proteins, such as division-specific transglycosylases and transpeptidases (11, 12). Together, these proteins synthesize the bacterial peptidoglycan cell wall. The inward-growing cell wall eventually separates the two daughter cells. Importantly, cell wall synthesis during cytokinesis is coupled with DNA replication and segregation, ensuring the genomes are faithfully segregated into daughter cells (13, 14). In rod-shaped model bacteria like *Escherichia coli* and *Bacillus subtilis*, division site selection is governed by two protein systems (15-17): nucleoid occlusion and the Min system. The nucleoid occlusion system prevents cytokinesis and septum closure over the condensed DNA within the nucleoid (18). In *E. coli*, the nucleoid occlusion protein SlmA protein binds to the nucleoid, inhibiting FtsZ polymerization (19-21). In *B. subtilis*, the unrelated ParB-like protein Noc conveys the nucleoid occlusion effect (22-25).

The second division regulating system is the Min system. The Min system was identified by the formation of small, round, anucleate cells that seemingly divided close to the cell poles (26). The gene locus responsible for the “miniature” cell phenotype in *E. coli* was termed *minB* (27, 28). Sequence analysis later revealed that the *minB* locus contains three structural genes, *minC*, *minD* and *minE* (29). MinC is the actual inhibitor of FtsZ polymer formation (30, 31). The MinC protein contains an N- and C-terminal domain (32) and both have been implicated with FtsZ inhibition. The C-terminal domain of MinC binds to the C-terminal tail of FtsZ and the N-terminal domain of MinC (33, 34), which is also responsible for dimerization, binds at the interface of two FtsZ subunits (35). MinC mediated inhibition of FtsZ was also confirmed in *B. subtilis* (36-38), suggesting a similar mechanism for cell division regulation in *E. coli* and *B. subtilis*.

The spatial localization of MinC is regulated by MinD, a Walker-type ATPase that belongs to the ParA/MinD family of proteins (36, 39, 40). These partitioning ATPases are involved in positioning cargo within bacterial cells (41, 42). While the molecular details of partitioning and positioning differ among various ParA-like proteins, a common factor is that they use binding to a large surface within the bacterial cell as a template for their positioning activity (41, 42). ParA proteins bind non-specifically to DNA, covering the nucleoid and exploiting it as a spatial track for positioning their cargo (43). In contrast, MinD proteins bind to the membrane surface via an amphipathic helix (44, 45). Membrane binding of *E. coli* MinD depends on ATP binding and subsequent dimerization (39). Dimerized MinD recruits MinC and activates it to prevent FtsZ dynamics (46). In *E. coli*, MinE further regulates MinD localization. MinE binds to the membrane and interacts with MinD, stimulating ATP hydrolysis, which leads to the release of MinD from the membrane. Slow nucleotide exchange within cytosolic MinD leads to an oscillatory cycle of MinD binding and detachment, resulting in a pole-to-pole oscillation of the MinCD proteins (47–49). This oscillatory behavior creates a temporal minimum of MinC molecules at the precise cell center, thus licensing this area for FtsZ ring condensation and cell division. This oscillatory behavior is crucial for proper cell division and can be reproduced *in vitro* on planar membranes, demonstrating the robustness of this self-organizing system (50).

In the Gram-positive model bacterium *B. subtilis* homologs of MinC and MinD are encoded (51, 52). Deletion of the respective genes also leads to minicell formation and hence, a role for the Min system similar to the case in *E. coli* has been proposed for *B. subtilis* (36, 53). However, a homolog of MinE is missing in *B. subtilis* and cell biological observation revealed that the *B. subtilis* Min system does not oscillate from pole to pole (36, 53). Rather, static fluorescence imaging revealed an enrichment of MinD at the cell poles and at active division sites. Early genetic and cell biological analysis revealed that the polar scaffold protein DivIVA is required for polar MinD localization in *B. subtilis* (36). The interaction of DivIVA and MinD is mediated by the intermembrane protein MinJ (54, 55). MinJ was also shown to be part of the divisome and interacts with several division proteins (55). MinJ also seems to play a role in divisome disassembly, since proteins such as FtsL and PBP2b remain associated with the young cell pole after division completion in mutant strains lacking MinJ (56). This function of the *B. subtilis* Min system is also supported by recent findings that MinD is involved in FtsZ polymer disassembly at the cell poles and deletion of *minD* inhibits polar peptidoglycan remodeling (57).

We have recently shown that all component of the *B. subtilis* Min system, including DivIVA and MinJ dynamically localize within the bacterial cell (56, 58–60). Interestingly, even the scaffold protein DivIVA, which is highly conserved in many Gram-positive phyla, is not static associated with the cell pole, but dynamically redistributes along the plasma membrane from poles to septa (58–60). The dynamics of closely related DivIVA species in actinobacteria show a drastically reduced mobility, in line with their function in apical growth (59). Therefore, a long-standing question in the field was why the *B. subtilis* Min system retained its dynamic localization. In particular, the role of the *B. subtilis* MinD ATPase cycle remained elusive, and the field has been searching for a potential ATPase-stimulating protein that could replace the role of the *E. coli* MinE protein for years. To address this question, we performed an in-depth *in vitro* analysis of *B. subtilis* MinD and studied its ATP hydrolysis and membrane-binding kinetics. Surprisingly, we found that the stimulation of ATP hydrolysis requires only MinD membrane binding, with no need for an additional activating protein. Furthermore, studies with various MinD mutant variants showed that *B. subtilis* MinD binds to the membrane in both its monomeric and dimeric forms, in contrast to the *E. coli* MinD homolog. We confirmed these findings *in vivo* using state-of-the-art single-molecule localization microscopy (SMLM) and single-molecule tracking (SMT). MinD dimers seem to be stabilized by MinJ at cell poles and the site of ongoing division, as judged by average localization of mutants using SMLM. Importantly, all fusion proteins were expressed as allelic replacements, resulting in native copy numbers of the analyzed proteins, a prerequisite for

precise *in vivo* dynamics determination. This work reveals that the *B. subtilis* Min system does not require any unidentified components. Min dynamics can be fully explained by the current data, providing new insights into the mechanisms of cell division in *B. subtilis*.

Results

Since localization and function of MinD appear to be strongly tied to its ATPase activity, the first logical step in describing and understanding details of the ATPase cycle was identification of the relevant biochemical characteristics, to eventually be able to correlate these properties to *in vivo* observations.

Purification and gel filtration of His-MinD

To this end, *B. subtilis minD* was cloned into a pET28a expression vector to obtain an N-terminally His-tagged fusion protein, since C-terminal MinD fusions have been shown to interfere with membrane binding (61). Recombinant His-MinD was then purified from the soluble fraction of *E. coli* lysate (Fig. S 1). Since MinD is notorious for being membrane-associated and thus complicating the purification effort, a well-established *E. coli* MinD purification protocol was adapted (62), where buffers were supplied with excess Mg^{2+} -ADP to potentially reduce MinD membrane interaction. Purification included Ni-NTA affinity chromatography followed by gel filtration of the pooled, concentrated sample. Based on the gel filtration chromatogram, His-MinD is still able to dimerise, as evidenced by two elution peaks corresponding to the expected sizes of the monomeric (~31 kDa) and dimeric forms (~62 kDa, Fig. S 1 and Fig. S 2). Furthermore, the ability of His-MinD to form larger oligomers or aggregates was suggested by western blotting, where presence of His-MinD in minor peaks of faster eluting fractions could be observed (Fig. S 1). Unfortunately, the Mg^{2+} -ADP excess in the purification buffers did not seem to stop His-MinD membrane interaction, as a significant fraction of the recombinant protein were later detected to be lost in the cell pellet. However, sufficiently high His-MinD concentrations could be obtained to proceed with biochemical analysis.

B. subtilis MinD displays full ATPase activity when incubated with ATP and liposomes

Being able to purify His-MinD allowed us to investigate the biochemical conditions affecting the MinD ATPase cycle next. For this purpose, we choose to use the commercially available enzyme coupled EnzChek™ phosphate assay kit (Thermo Fisher Scientific). Briefly, it allows a rapid and sensitive detection of phosphate released during ATPase reactions. In the presence of inorganic phosphate, a purine nucleoside phosphorylase (PNP) converts a substrate (MESG) into a product exhibiting a spectrophotometric shift in maximum absorption from 330 nm to 360 nm, allowing a colorimetric determination of free phosphate levels in an effective range between 2 and 150 μ M.

We determined the ATPase activity by measuring the P_i production rate (in μ M min^{-1}) as a function of His-MinD concentration. When we first tested His-MinD by itself at concentrations between 0.5 μ M and 16 μ M, however, only baseline activity below the detection limit could be observed (data not shown). Since MinD interacts with the membrane and it was expected that the ATPase cycle is involved in controlling this interaction, we next measured ATPase activity in the presence of liposomes extruded from *E. coli* total lipids (Avanti). Remarkably, this led to a robust increase in ATPase activity of His-MinD, appearing to be linearly dependent on protein concentration (Fig. 1 A & B). To be able to compare the activity level to the published *E. coli* MinD *in vitro* ATPase data (63–65), the specific activity is expressed in $\text{nmol mg}^{-1} \text{min}^{-1}$ (Fig. 1 B). To our surprise, activity levels of recombinant *B. subtilis* MinD were found to be on par with fully active *E. coli* MinD in conjunction with MinE and phospholipids (63). Moreover, the ATPase activity did not show cooperative behavior compared to *E. coli* MinD at lower

concentrations (63 [C](#)). We further measured that ATP hydrolysis can be titrated with both ATP and liposomes and is described by Michaelis-Menten kinetics (**Fig. 1** [C](#) & [D](#)). Again, the hereby-obtained $V_{\max}$ values (19.50 nmol mg⁻¹ min⁻¹ and 22.05 nmol mg⁻¹ min⁻¹, respectively) indicate similar MinD ATPase activity levels between both organisms.

MinD ATPase activity is triggered by membrane binding

To be able to test if membrane-interaction itself is the catalyst for MinD ATPase activity, the need for a membrane-binding mutant was evident. MinD interacts with the membrane through an amphipathic α -helix located at the N-terminus, thus being the membrane targeting sequence (MTS) (**Fig. 2** [A](#)). Exchanging the hydrophobic isoleucine residue in the apolar core of the MTS with a charged glutamate (I260E) has been shown to abolish membrane binding of MinD by perturbing the amphipathic properties, demonstrated through fluorescence microscopy (66 [C](#)). Accordingly, a His-MinD-I260E variant was constructed and purified via the established purification protocol. The yield of the purification was extensively higher compared to the wild type protein (Fig. S 3), hinting towards a successful abolishment of membrane interaction.

Moreover, as we wanted to be able to compare *in vivo* and *in vitro* data, a *B. subtilis* strain for microscopy was constructed, where the native copy of MinD was replaced by a HaloTag-MinD fusion protein (**Fig. 2** [B](#), HaloTag will henceforth be referred to as Halo). We then introduced the I260E mutation, resulting in a strain producing Halo-MinD-I260E as the only copy. Microscopic analysis of a Halo-MinD-I260E with TMR dye confirmed drastically reduced membrane binding through its almost exclusive cytosolic localization (**Fig. 2** [B](#)).

We next repeated ATPase activity assays with the purified, recombinant I260E mutant, and remarkably, activity remained below the detection limit of the assay, while a positive control of His-MinD wild type protein displayed regular activity (Fig. S 4). In summary, these data collectively suggest that membrane binding is necessary and the key trigger for ATP hydrolysis of *B. subtilis* MinD, without the apparent need for another stimulus.

This conclusion arose new questions that needed to be answered in order to understand the ATPase cycle of MinD in *B. subtilis*. Membrane binding of MinD in *E. coli* is primarily possible for the ATP bound, dimeric form, but if that were the case in *B. subtilis*, can dimers only form in the cytosol? Alternatively, can *B. subtilis* MinD bind the membrane as monomers, so that the membrane could act as a proxy for dimerisation of MinD?

Construction of catalytic MinD mutants

To be able to answer these questions, we explored further mutations that affect the ATP binding region and thus the dimerization process. Due to the conserved nature of Walker-type MinD/ParA-like ATPases, residues involved in catalytic activity have been well characterized so that different ATPase mutants have been established (67 [C](#)-69 [C](#)). We therefore constructed expression plasmids encoding three different MinD variants (illustrated in **Fig. 2** [A](#)): This first mutation, MinD-G12V, produces a steric clash within the active site, so that ATP can still be bound, but dimers cannot form (67 [C](#), 68 [C](#)). The second mutation, K16A, completely disrupts ATP binding and hence yields another monomeric mutant (67 [C](#)). The third variant encodes the mutation D40A, which should interfere with ATP hydrolysis (68 [C](#), 69 [C](#)). Therefore, it was expected that MinD-D40A forms a trapped nucleotide sandwich dimer similar to Soj or *E. coli* MinD (68 [C](#), 69 [C](#)). All MinD variants were successfully expressed and purified with the previously established protocol and resulted in similar protein yields when compared to the wild type protein (Fig. S 1, Fig. S 5, Fig. S 6 and Fig. S 7). When evaluating the gel filtration chromatograms, His-MinD-G12V elute in a single peak, which can be interpreted as a monomer when compared to the wild type His-MinD elution peak (Fig. S 2). His-MinD-D40A on the other hand also eluted in a single peak, albeit being wider and eluting earlier, indicating a mainly dimeric conformation. Only His-MinD-

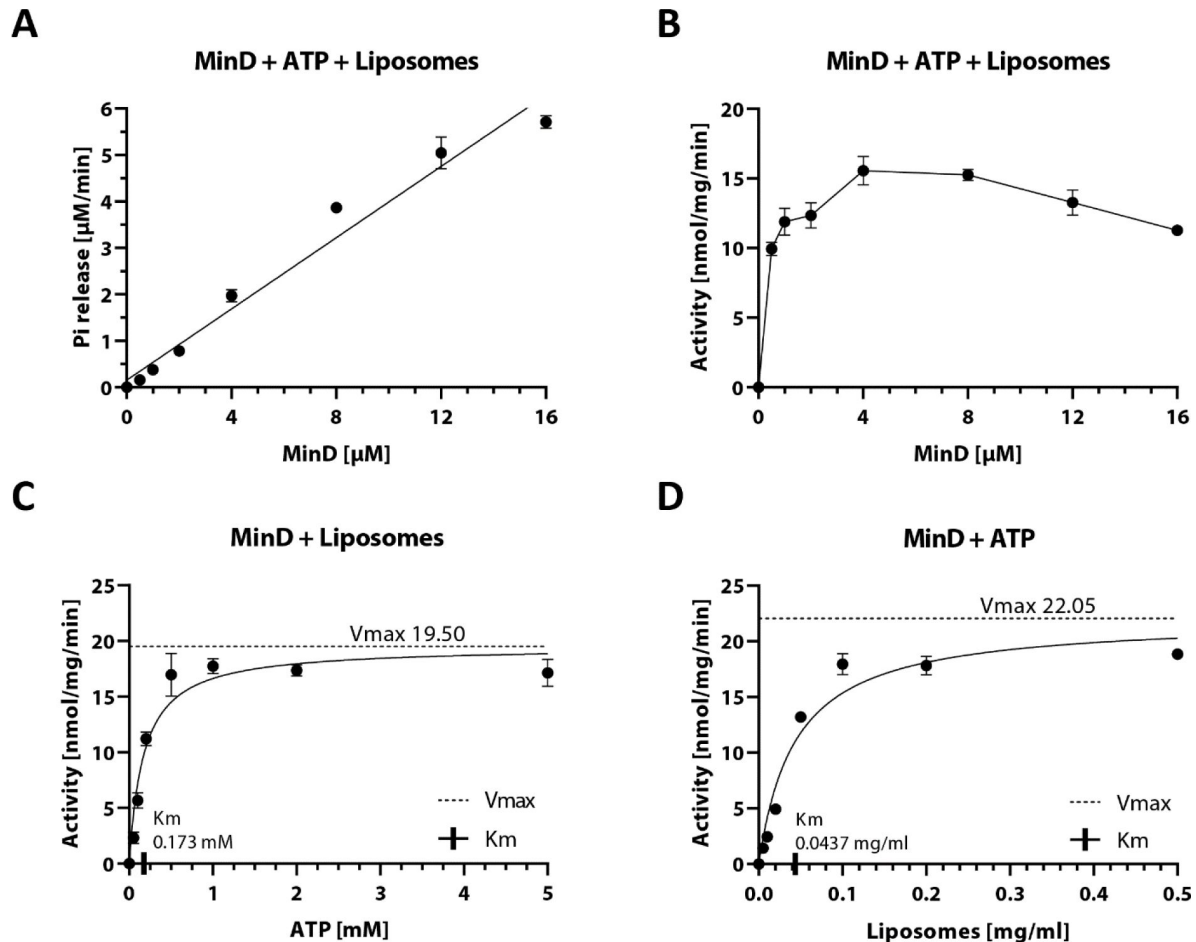


Fig. 1

Biochemical analysis of *B. subtilis* MinD ATPase cycle.

(A) Phosphate release plotted against different MinD concentrations, fitted with a simple linear regression ($R^2 = 0.97$).

Phosphate release was measured using the EnzChek™ phosphate assay kit. Samples contained 0.2 mg ml^{-1} liposomes and were pre-incubated for 10 minutes before addition of 2 mM Mg^{2+} -ATP; $n \geq 3$.

(B) Specific activity of the MinD ATPase from (A) measured as a function of MinD concentration.

(C) Specific activity of MinD measured as a function of ATP concentration, determined as in (B) with fixed MinD concentration of $10 \mu\text{M}$. Fitting the Michaelis-Menten equation (black lines) gives $k_{\text{cat}} = 36.27 \text{ h}^{-1}$, $V_{\text{max}} = 19.50 \text{ nmol mg}^{-1} \text{ min}^{-1}$ and $K_M = 0.173 \text{ mM}$.

(D) Specific activity of MinD measured as a function of liposome concentration, determined as in (B) with fixed MinD concentration of $6 \mu\text{M}$. Fitting the Michaelis-Menten equation (black lines) gives $k_{\text{cat}} = 41.01 \text{ h}^{-1}$, $V_{\text{max}} = 22.05 \text{ nmol mg}^{-1} \text{ min}^{-1}$ and $K_M = 0.0437 \text{ mg}/\text{ml}$.

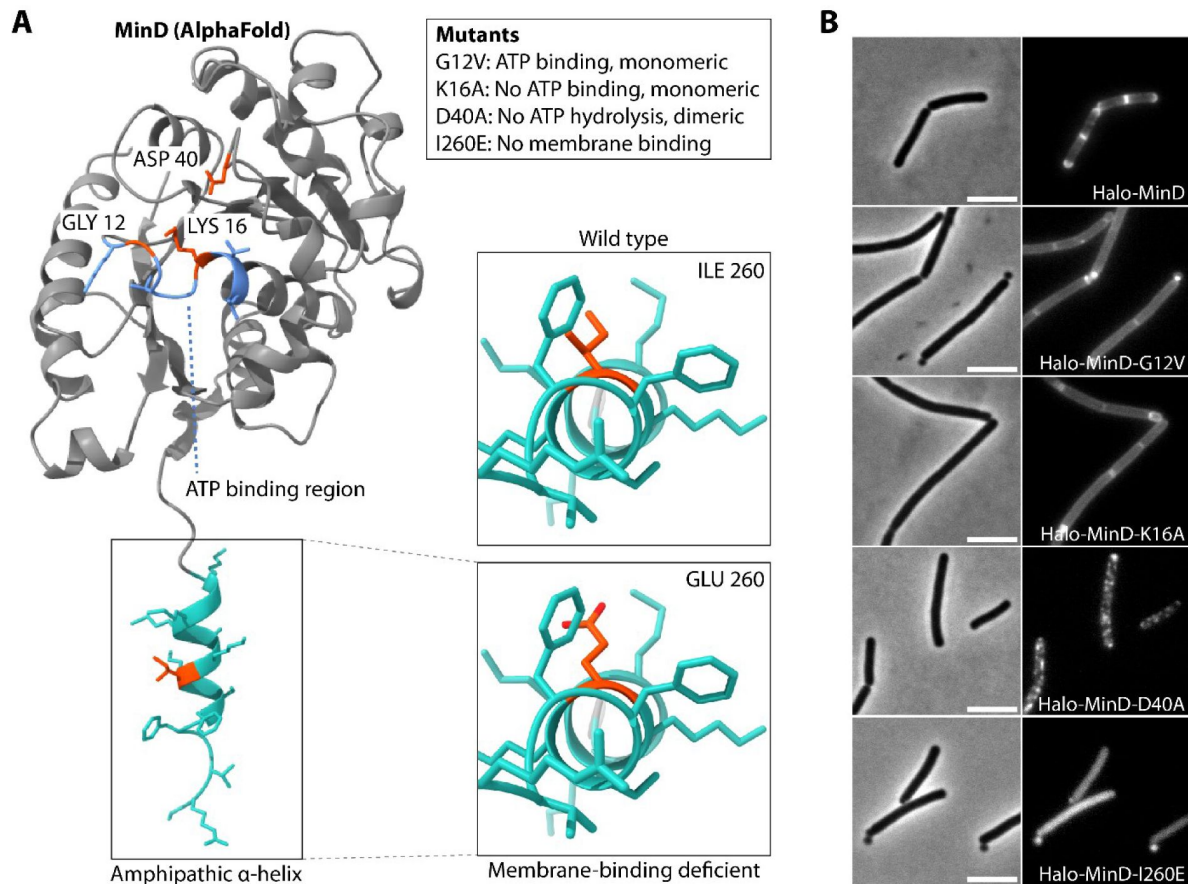


Fig. 2

Cartoon of MinD AlphaFold model with highlighted key amino acids and the effects of their mutagenesis.

(A) Left: Rendering of MinD AlphaFold model with ATP binding region highlighted in light blue, membrane targeting sequence (MTS) in turquoise and key residues in orange; Box: explanation of mutagenesis effects. Right: Close-up on hydrophobic region of amphipathic helix.

(B) Representative wide-field microscopy images of *B. subtilis* expressing indicated Halo-MinD variants as the only MinD copy from the native locus, stained with TMR ligand. Left: Phase contrast; scale bar 5 μ m. Right: fluorescence.

K16A reproducibly formed a rather unexpected shoulder in its main elution peak, which could hint towards further interaction with the gel filtration matrix or other potential self-interactions. As expected, all of the recombinant His-MinD mutants were catalytically inactive when tested via the EnzChek phosphate assay kit.

To investigate *in vivo* effects of these variants, the same mutations were introduced into the strain expressing Halo-MinD in *B. subtilis*, respectively, and subsequently imaged via wide-field microscopy (Fig. 2B). Surprisingly, both monomeric MinD variants (G12V and K16A) were still observed to be membrane associated, however resulting in the apparent loss of the polar-septal MinD gradient (Fig. 2B). All strains producing MinD mutants manifested an increased cell length and minicell production, as it was expected in cells containing dysfunctional Min proteins. Finally, the locked dimer D40A mutant appeared to be mainly membrane associated, thereby forming large foci indicating accumulation of MinD, but much less dispersed when compared to G12V or K16A (Fig. 2B). In summary, the time-resolved MinD gradient appears to be lost in all MinD mutants, including D40A. This suggests that the ATPase cycle and thus quick cycling of MinD is indeed necessary for formation of the observed dynamic gradient, as it has been previously suggested (60).

Monomeric MinD binds the membrane with high efficiency

Membrane binding appeared to be a key factor of the ATPase cycle and since the microscopy-data indicated binding of MinD monomers to the membrane, we next aimed to determine biochemically, if the respective MinD variants are capable of binding to membrane and if so, to characterize their respective binding kinetics. To this end, we employed Bio-layer interferometry (BLI), a technique capable of quantifying binding strength and protein interaction, as well as identifying reaction kinetics.

As we were interested in MinD-membrane interaction, the first step was to immobilize liposomes on a biosensor to create a membrane-like surface. Therefore, *E. coli* total lipids (Avanti) were extruded after mixing in a ~33:1 ratio with DSPE-PEG₍₂₀₀₀₎-biotin (Avanti), a biotinylated polyethylene glycol lipid extending from the liposomes and able to bind Streptavidin (SA) coated biosensors (70, 71). After successful saturation of the sensor with liposomes, MinD-liposome association and dissociation could be measured, a scheme of the experimental set-up is depicted in Fig. 3A. As a negative control for unspecific binding, MinD-sensor interaction was tested on empty SA sensors. Furthermore, unspecific binding to liposome-saturated sensors was tested using bovine serum albumin (BSA). Both resulted in negligible, diffuse signal (data not shown).

Kinetics of His-MinD binding were tested first, using different protein concentrations between 1 μM and 16 μM in presence of ATP. Binding to the sensor-bound liposomes scaled with increasing protein concentration, resulting in an equilibrium dissociation constant $K_D = 7.17 \mu\text{M}$ and an association rate constant $k_{\text{on}} = 3.03 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ as a reference point for the wild type protein (Fig. 3B, Tab. 1). Unsurprisingly, His-MinD-I260E showed a more than 10-fold reduced affinity to the membrane with a $K_D = 74.36 \mu\text{M}$ (Tab. 1), which is also apparent in the binding graph (Fig. 3B). The strong increase in K_D is based on the over 16-fold lowered association $k_{\text{on}} = 0.18 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$, while the dissociation $k_{\text{off}} = 13.45 \times 10^{-3} \text{ s}^{-1}$ was also slightly lower when compared to wild type MinD. Next, both monomeric mutants G12V and K16A were probed, and to our surprise, capable of membrane interaction (Fig. 3C & D, Tab. 1). This is in stark contrast to *E. coli* MinD, where dimerization needs to precede membrane binding to achieve sufficient affinity (44, 45, 64, 69, 72). Similar to I260E, the G12V mutant displayed an around 10-fold reduced membrane affinity equilibrium with $K_D = 75.38 \mu\text{M}$ (Tab. 1). However, G12V only exhibited a ~5 fold reduced binding affinity with $k_{\text{on}} = 0.63 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ when compared to the wild type MinD, while the $k_{\text{off}} = 47.8 \times 10^{-3} \text{ s}^{-1}$ represents the fastest dissociation rate constant between all MinD variants and more than 2-fold increase compared to wild type MinD (Tab. 1). This indicates a higher turnover and thus a quicker release of the protein from the membrane. Markedly different from this, K16A displayed a K_D of 1.36 μM , but not much lower binding rates

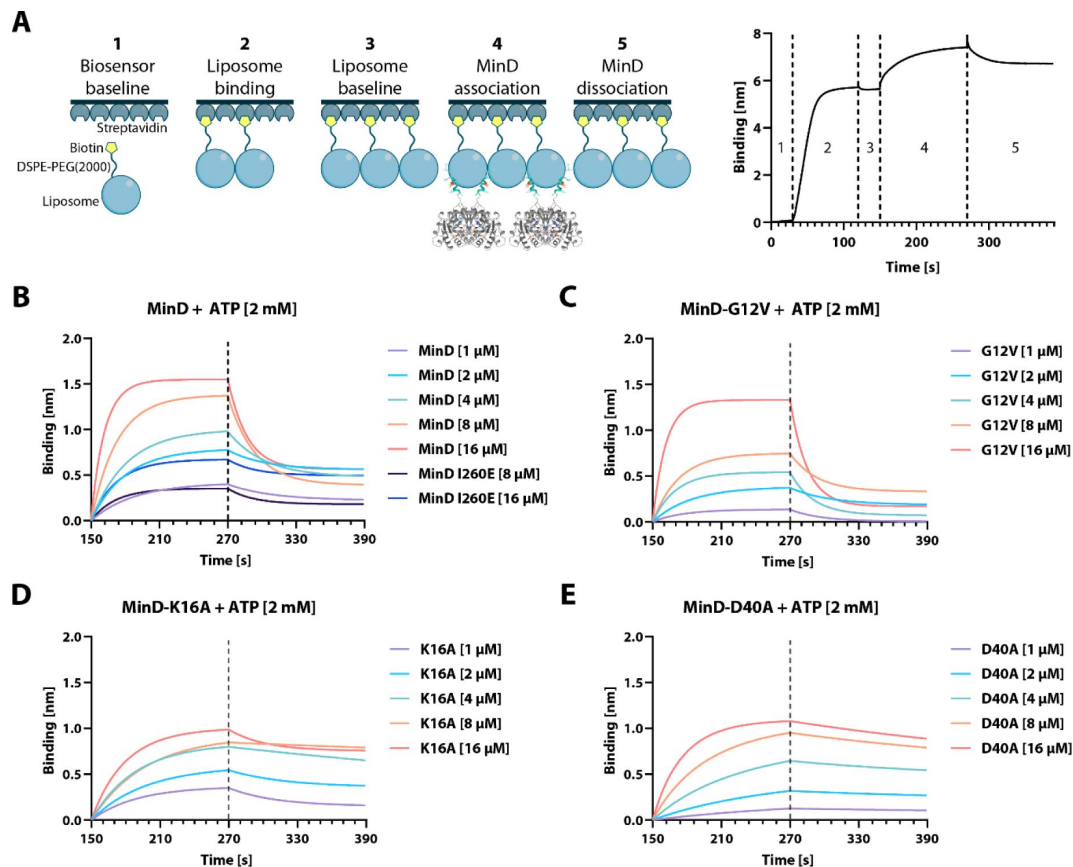


Fig. 3

BLI analysis of His-MinD and different mutants.

(A) Left: Cartoon representation of the different BLI steps, starting with (1) establishing a baseline in protein buffer, (2) binding of liposomes through the biotinylated DSPE-PEG(2000) phospholipid, (3) establishing a new baseline, (4) binding of MinD and finally (5) dissociation of MinD. Right: Exemplary graph resulting from sensor readouts of steps (1)-(5). Scheme adapted from (70).

(B) MinD binding plotted against time through association and dissociation phase (steps 4-5) at different protein concentrations, including the His-MinD-I260E mutant.

(C) – (E) Same as (B) using the indicated respective mutant of His-MinD (G12V, K16A and D40A).

($k_{\text{on}} = 2.03 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$, **Tab. 1** & **Fig. 3** C). Instead, the low K_D can be mostly attributed to the reduced dissociation ($k_{\text{off}} = 2.77 \times 10^{-3} \text{ s}^{-1}$), implying a much longer retention of His-MinD-K16A at the membrane (**Fig. 3** D). Since G12V can bind ATP while K16A cannot, these findings reveal that presence of ATP in the binding pocket affects association and dissociation of MinD.

Last, the His-MinD-D40A mutant protein was probed, resulting in an almost 10-fold reduced dissociation equilibrium $K_D = 0.74 \text{ } \mu\text{M}$ (**Tab. 1**, **Fig. 3** E). Here, the strongly reduced membrane dissociation ($k_{\text{off}} = 1.76 \times 10^{-3} \text{ s}^{-1}$) causes the drastic change in K_D , which we expected for the trapped dimer, as it could position the two amphipathic helices into a more optimal orientation of the apolar residues towards the membrane, resulting in stronger binding and no capability of hydrolyzing ATP and thus relieving the dimer (66, 68, 69).

In summary, these BLI data demonstrate that *B. subtilis* MinD is capable of binding membrane in both monomeric and dimeric form, while the presence of ATP in the binding pocket appears to affect both membrane binding and dissociation.

Single-molecule localization microscopy confirms *in vitro* data

To validate our findings, which were based on biochemical *in vitro* results, we decided to utilize single-molecule localization microscopy (SMLM) with focus on single-molecule tracking (SMT), a technique allowing to observe and evaluate MinD dynamics *in vivo* inside living *B. subtilis* cells. For this reason, the previously introduced strains expressing Halo-MinD and variants (Tab. S 3), respectively, were grown to mid-exponential phase, stained with TMR and subsequently transferred and imaged on agar pads supplemented with medium. Every field of view (FOV) was imaged for 10,000 frames of 24 ms each, and individual fluorescence localizations were either fitted with the Zen Black software (Zeiss) and analyzed with individual R scripts for localization data (**Fig. 4** A), or TrackMate and the SMTracker 2.0 software packages to build protein trajectories and to analyze mobility and dynamics (**Fig. 4** B-E) (73-76).

We began SMLM analysis by plotting a heat-map of the intracellular localization of all individually recorded Halo-MinD molecules as well as the mutated variants, respectively, on normalized cells (**Fig. 4** A). This representation did not only provide a robust internal control for the imaging and post-processing process for the well characterized localization of MinD, but also reveals systemic differences in localization caused by the respective mutations. The first obvious difference is the lower local enrichment of MinD at the cell center and poles when carrying the G12V or K16A mutation (**Fig. 4** A). Here, the membrane-bound MinD fraction seems to be much more evenly distributed along the membrane, similar to what we observed during wide-field microscopy (**Fig. 2** B). This result does not only confirm the ability of MinD monomers to bind the membrane (**Fig. 3** C & D), but also fuels the speculation that MinJ might be unable to recruit MinD in its monomeric form, so that polar and septal recruitment by MinJ requires dimeric MinD. In combination with the information obtained from BLI results (**Fig. 3**), this could mean that the membrane itself serves as proxy for MinD dimerisation and subsequent recruitment, quickly followed by ATP hydrolysis and membrane detachment of MinD. In contrast, but in agreement with wide-field imaging (**Fig. 2** B), the Halo-MinD-D40A mutant does appear to frequently form foci and larger local accumulations, with little protein in the cytosolic fraction (**Fig. 4** A). This, again, could be caused by local MinJ interaction and recruitment in combination with the missing capability of membrane detachment or with increased lateral MinD interactions (77). Finally, the I260E mutant appears evenly distributed throughout the cell (**Fig. 4** A), as it was expected.

Independently, all protein trajectories were subjected to a stationary localization analysis (SLA) (**Fig. 4** B). This was done by first grouping all trajectories into an either mobile or static population by testing if the molecule left a circle with a diameter of 97 nm (equaling the pixel size of the imaging setup) within at least five consecutive frames. Trajectories that were classified as mobile were further sub-grouped into mixed and free trajectories, where only free trajectories never presented a confinement event (73). In agreement with previous data, the D40A mutant

Protein	K_D [μM]	k_{on} [$\text{M}^{-1} \text{s}^{-1}$]	$k_{on \text{ error}}$	k_{off} [s^{-1}]	$k_{off \text{ error}}$	χ^2	R^2
MinD	7.17	3.03×10^3	0.13×10^3	21.7×10^{-3}	0.33×10^{-3}	16.07	0.95
MinD-G12V	75.38	0.63×10^3	0.19×10^3	47.8×10^{-3}	1.13×10^{-3}	13.89	0.95
MinD-K16A	1.36	2.03×10^3	0.062×10^3	2.77×10^{-3}	0.088×10^{-3}	6.966	0.95
MinD-D40A	0.74	2.39×10^3	0.053×10^3	1.76×10^{-3}	0.069×10^{-3}	6.006	0.98
MinD-I260E	74.36	0.18×10^3	0.19×10^3	13.45×10^{-3}	0.38×10^{-3}	3.607	0.95

K_D = equilibrium dissociation constant; k_{on} = association rate constant; k_{off} = dissociation rate constant

Tab. 1

Kinetic constants of His-MinD and variants obtained via BLI.

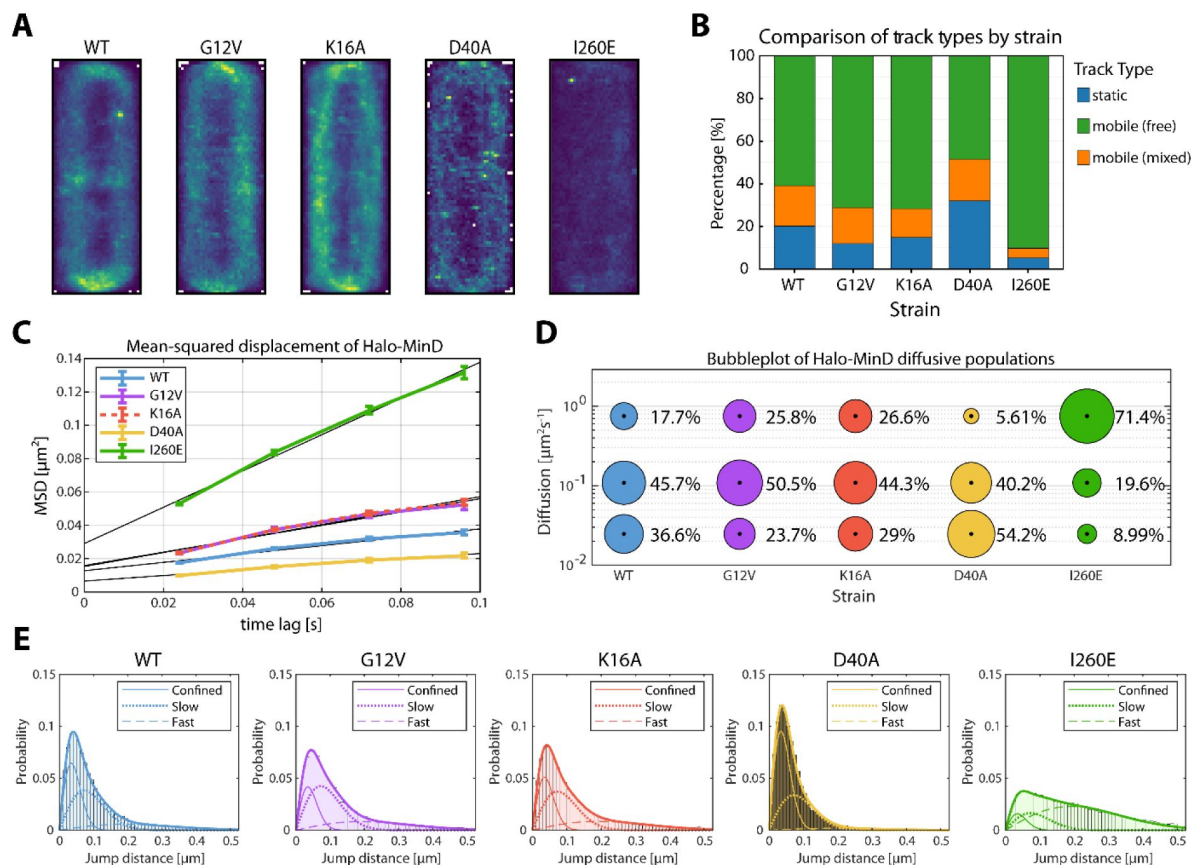


Fig. 4

Single-molecule localization microscopy analysis of Halo-MinD and mutants expressed in *B. subtilis*.

Exponentially growing *B. subtilis* cells expressing Halo-MinD and variants ($n \geq 48$ cells, respectively) were stained with TMR ligand and subsequently imaged. Individual protein trajectories were recorded using SMLM and analyzed with Zen blue (Zeiss), Trackmate, the SMTracker 2.0 software package and manual scripts in R. Minimum track-length 4 frames of 24 ms, with at least 2596 trajectories per strain.

(A) Heat map representation of intracellular localization of individual molecules of Halo-MinD and variants, respectively, plotted on normalized cells. Brighter colors indicate higher abundance.

(B) Barplot of stationary localization analysis (SLA), comparing different track types within the protein population. Tracks were considered static when not leaving a circular area of 97 nm diameter within 5+ frames. Mobile populations were further divided into free and mixed tracks, where mixed tracks displayed a switch between free and confined movement.

(C) Plot of the mean-squared displacement of Halo-MinD and variants over time, fitted with a linear fit excluding the last timelag.

(D) Bubble plot displaying single-molecule diffusion rates of the indicated MinD fusions. Populations were determined by fitting the probability distributions of the frame-to-frame displacement (jump distance) data of all respective tracks to a three components model (fast mobile, slow mobile and confined protein populations).

(E) Probability distributions of jump distances of Halo-MinD and variants. Data was fit with a three component model, indicating confined, slow and fast tracks.

displayed the highest fraction of static tracks (**Fig. 4** [B](#)), and by far the lowest proportion of truly free trajectories (48.5 %). In contrast, more than 90% of Halo-MinD-I260E molecules were classified as free with only few trajectories classifying as static (**Fig. 4** [B](#)), accentuating the absence of interaction with the membrane or other structures. When comparing Halo-MinD to the monomeric mutants G12V and K16A, the number of static tracks was visibly reduced in the mutants. Since both monomers are unable to dimerise, it is less likely that they can form larger clusters, which we observed in a previous study ([60](#) [B](#)). Moreover, interaction with MinJ stabilizes MinD ([60](#) [B](#)), and the possible abolishment of this stabilization should also result in a mobilizing effect. Between both monomeric mutants, K16A displayed a slightly larger number of static trajectories (**Fig. 4** [B](#)), which is in alignment with BLI data, where the K16A mutant dissociates much less effectively from the membrane (**Fig. 2** [C](#) & **D**, **Tab. 1** [B](#)).

Next, we wanted to learn about the average intracellular mobility of Halo-MinD in comparison to the mutated variants, which prompted us to analyze the mean-squared displacement (MSD, **Fig. 4** [C](#)). In MSD analysis, the traveled distances of recorded trajectories are plotted against a given time lag (Δt) as a function of Δt , here one imaging frame (24 ms), where the slope can indicate the speed ([78](#) [B](#)–[80](#) [B](#)). As expected, the I260E mutant displayed the fastest average mobility, since it does not bind the membrane effectively anymore (**Fig. 4** [C](#)). In contrast, the D40A mutant was the slowest recorded protein, indicated by the only mild incline (**Fig. 4** [C](#)). Since we expect most of the proteins' population to be membrane bound, and furthermore observed larger foci that suggest cluster formation, this was expected. Halo-MinD wild type protein appeared to be more mobile in comparison (**Fig. 4** [C](#)), as it should exist in both monomeric and dimeric conformation and either freely diffusive in the cytosol or interact with the membrane. Last, the two monomeric mutants G12V and K16A displayed extremely similar average speeds that were both faster than the wild type protein (**Fig. 4** [C](#)). Since these MinD variants cannot form dimers, general diffusion should be faster, as there should be no MinD dimers or oligomers, and possibly no recruitment by MinJ.

If the underlying movement in MSD analysis can be described by simple Brownian motion, the gradient of the curve is proportional to the diffusion coefficient D ([80](#) [B](#)). We know, however, that MinD interacts at least with MinC, MinJ, the membrane and itself, in combination with the fact that bacterial cells exhibit natural confinement. With these information one cannot assume simple Brownian motion, therefore we have chosen jump distance (JD) analysis as a more in-depth assessment to describe Halo-MinD dynamics (**Fig. 4** [D](#) & **E**). In JD analysis, the distances molecules travel between subsequent frames are plotted in a probability distribution (**Fig. 4** [E](#)), and diffusion coefficients are obtained by curve fitting ([80](#) [B](#)), in this case with a three-component model separating fast, slow and confined molecules (**Fig. 4** [D](#) & **E**). To be able to compare population sizes better, the simultaneous option was chosen in the SMTracker software, fixing the diffusion coefficients for every MinD variant ([73](#) [B](#)). To also obtain individual, variant specific diffusion coefficients, the same data was additionally fitted individually (non-simultaneous SQD analysis in SMTracker2, **Tab. 2** [B](#)).

Generally, the results of JD analysis correlated well with our *in vitro* data. When dissecting the different populations in the wild type, more than a third of all proteins appear to be confined (36.6%, **Fig. 4** [D](#)), which likely comprises membrane bound protein that is further stabilized by protein-protein interactions via lateral interaction of MinD with MinC and MinJ. The slow mobile population was the largest (45.7%, **Fig. 4** [D](#)), and possibly summarizes different conformations of MinD that interact with the membrane, but are still diffusive and not captured or stabilized through further interactions. The third, fast diffusive population (17.7%, **Fig. 4** [D](#)) likely recapitulates cytosolic MinD. These results stress the importance of the MinD ATPase cycle for its localization and dynamics, as these are severely altered in the MinD mutants.

Strain	Fast diffusive [$\mu\text{m}^2 \text{s}^{-1}$]	Slow diffusive [$\mu\text{m}^2 \text{s}^{-1}$]	Confined [$\mu\text{m}^2 \text{s}^{-1}$]
MinD WT	0.60 (22.3 %)	0.084 (50.4 %)	0.020 (27.3 %)
MinD-G12V	0.68 (28.5 %)	0.10 (47.5 %)	0.026 (24.0 %)
MinD-K16A	0.70 (27.3 %)	0.11 (43.9 %)	0.024 (28.8 %)
MinD-D40A	0.44 (13.7 %)	0.063 (50.0 %)	0.021 (36.3 %)
MinD-I260E	0.76 (73.4 %)	0.095 (20.3 %)	0.022 (6.3 %)

Percentages indicate relative population size

Tab. 2

Diffusion coefficients obtained from non-comparative SQD analysis of Halo-MinD and variants fitted with three populations.

In detail, the probability distributions of Halo-MinD-D40A and I260E (**Fig. 4** [E](#)) illustrate the strong shift between MinD molecules in a confined (D40A) and a diffusive state (I260E) best. When examining the different populations, most Halo-MinD-D40A molecules were classified as confined (54.2%) and slow mobile (40.2%), whereas I260E only displayed ~9% of confined molecules (**Fig. 4** [D](#)), matching prior results and expectations of the membrane binding mutant. When comparing D40A to wild type Halo-MinD, the largest shift is observed between the fast mobile and confined populations, shifting towards a distinctively more confined regiment in the D40A mutant (36.6% WT, 52.2% D40A; **Fig. 4** [D](#)) and visible disappearance of the fast population in the probability distribution (**Fig. 4** [E](#)). These results are in line with the previous observations, as a trapped dimer of MinD will remain at the membrane at much higher efficiency (**Fig. 3** [E](#) & **Tab. 1** [A](#)), and appeared to form foci and clusters (**Fig. 2** [B](#), **Fig. 4** [A](#)), with the possibility of stabilization through MinJ. G12V and K16A mutants on the other hand showed an increase in fast mobile populations (WT 17.7%, G12V 25.8%, K16A 26.6%; **Fig. 4** [D](#) & [E](#)), which could be attributed to a reduced interaction with MinD interaction partners (MinC, MinJ and plasma membrane). This notion is supported by the decrease in confined molecules for both G12V and K16A mutants compared to the wild type (WT 36.6%, G12V 23.7%, K16A 29%; **Fig. 4** [D](#) & [E](#)), which are expected to be membrane bound and likely stabilized through other protein-protein interaction, which may be reduced or absent in the monomeric conformation. Finally, when comparing both monomeric variants, the K16A mutant displays a noticeable shift between the slow diffusive (G12V 50.5%, K16A 44.3%; **Fig. 4** [D](#)) and confined populations (23.7% G12V, 29% K16A; **Fig. 4** [D](#)). The larger confined population of Halo-MinD-K16A is consistent with the BLI results (**Fig. 3** [D](#), **Tab. 1** [A](#)), where K16A appears to have a lower dissociation from the membrane, increasing its likelihood of confinement, possibly caused by the inability to bind ATP.

In summary, these results emphasize the importance of the MinD ATPase cycle for functionality of the Min system in *B. subtilis*. Even though *B. subtilis* MinD is not capable of oscillation, its intracellular dynamics are highly dependent on the functionality of the MinD ATPase domain and its catalytic site, which is reflected by the variety of localization and interaction defects displayed by the MinD mutants on a molecular level, that all result in severe division anomalies.

Discussion

Pattern formation is a universal trait of living cells and tissues and a crucial factor in cellular self-organization ([81](#) [A](#)). This is best exemplified during embryonic development, where eukaryotic cells differentiate into various types that form specific tissues and organs. In particular, the formation of the body axis and the segmentation of the fruit fly embryo is governed by specific patterns that dictate the development of different body parts ([82](#) [A](#), [83](#) [A](#)). Disruptions in pattern formation leads to developmental abnormalities and diseases. Bacteria are single celled organisms, however, their morphogenesis also depends on subcellular pattern formation of morphogenic proteins that ensure correct synthesis of the cell components. These self-organizational process have been explained by the reaction-diffusion theory, first proposed by Alan Turing ([84](#) [A](#)). The beauty of the reaction-diffusion theory is that it can explain complex spatio-temporal organizations even in absence of a preexisting asymmetry. Thus, symmetry breaking is a key aspect of *de novo* pattern formation ([85](#) [A](#)). The model of the reaction diffusion theory is simply based on two components, an activator and an inhibitor, that interact chemically and diffuse at different rates in a given medium, such as the cytoplasm of a cell. Pattern formation is generated if one of the components diffuses differently to the other. Depending on the precise nature of the interaction and the diffusion properties, a variety of stable patterns can be generated by this process. In bacteria, the oscillation of MinCDE in *E. coli* is one of the finest examples of such a reaction-diffusion system, and the two core components MinD and MinE cycle between membrane and cytosol in an ATP-dependent manner, generating a standing wave with a time-resolved minimum at midcell ([15](#) [A](#), [86](#) [A](#)). Here, binding and unbinding of the components to the membrane surface influences the diffusion coefficients and controls the activation/inactivation of

the cell division inhibitor MinC (87 [↗](#)). The mechanism and dynamics of the *E. coli* MinCDE system have been subject of numerous studies and the system is therefore one of the best understood examples of intracellular pattern formation in bacteria (15 [↗](#), 87 [↗](#)-89 [↗](#)). In comparison, the dynamics of the Min system in *B. subtilis* have not been as well characterized. In this system, the activator of MinD ATP hydrolysis, MinE, is missing and therefore the MinD dynamics remained mechanistically unsolved. In a previous work we have characterized the subcellular dynamics of Min components in *B. subtilis* using FRAP analysis (60 [↗](#)). Although the scaffold protein DivIVA was long considered a static interaction hub at the cell poles and at growing septa, our data revealed that DivIVA is dynamically relocating within the cell, mainly associated in clusters at the cell membrane (58 [↗](#)-60 [↗](#)). Cluster formation was also observed for MinJ and MinD, with a clear enrichment of these clusters at the sites of septation. The observed dynamics explained how the entire Min system including DivIVA, MinJ, MinD and MinC quickly re-localizes from cell poles to midcell at the onset of cell division. Using the measured diffusion coefficients for the Min proteins in combination with their native protein numbers, a mathematical model was generated that suggested MinD protein gradient formation by a nonequilibrium process (60 [↗](#)). We suggested that gradient formation depends on the ATPase driven MinD dynamics. However, in spite of a lack in biochemical data, we had to assume that the *B. subtilis* MinD cycles between membrane-bound and cytosolic localizations, based on ATP-binding and hydrolysis, similar to the situation that is well established for the *E. coli* MinD (60 [↗](#)). A further gap in our knowledge of the *B. subtilis* Min system concerns the activation mechanism of the MinD ATPase in the absence of a MinE homolog. To fill this gap, we started to analyze the *B. subtilis* MinD protein biochemically *in vitro*. The isolated MinD protein revealed a basal ATPase activity that was stimulated by addition of liposomes (membrane). The *E. coli* MinD also requires lipid addition to stimulate ATPase activity, however, it also requires addition of the MinE protein for stimulation (63 [↗](#)). The maximum activity for the *E. coli* MinD was determined to be around 20 nmol mg⁻¹ min⁻¹ at conditions of full stimulation in presence of MinE and lipid (63 [↗](#), 64 [↗](#)). Surprisingly, the maximal activity of the *B. subtilis* MinD was almost precisely at the same velocity, indicating that *B. subtilis* MinD ATPase activity is triggered by membrane binding and may not require an additional protein factor such as MinE for its catalytic activation. When comparing to other MinD/ParA ATPases that have been biochemically characterized, the activity level of *B. subtilis* MinD is on the lower end of the spectrum with a measured k_{cat} of 36.27 h⁻¹. Soj, the ParA homologue in *B. subtilis*, reaches more than 2-fold higher catalytic activity (83.6 h⁻¹) when fully activated by supplementing Spo0J (ParB) and DNA (90 [↗](#)), similarly to Soj in *Thermus thermophilus* combined with Spo0J and *parS* containing DNA (68 [↗](#)). Studies investigating the activity levels of ParA in *Caulobacter crescentus*, purified in presence of ATP, determined a k_{cat} of 0.79 min⁻¹ (47.4 h⁻¹) in absence of ParB, increasing almost 5-fold to 3.7 min⁻¹ (222 h⁻¹) in presence of ParB (91 [↗](#)). A more recent study found the *C. crescentus* ParA k_{cat} increase from 5.8 hr⁻¹ to 120 hr⁻¹ upon addition of ParB (92 [↗](#)).

Thus, it is very plausible that the *B. subtilis* MinD does not need an additional protein partner that induces ATP hydrolysis. Rather, binding of MinD to the membrane itself is sufficient to stimulate ATP hydrolysis. While we cannot entirely rule out the possibility that interactions such as MinD binding to MinJ might further enhance nucleotide hydrolysis, the comparison of maximum velocities, activity stimulation factors, and K_M values between the *E. coli* and *B. subtilis* MinD proteins suggests that a functional homolog of the MinE protein is not necessary in *B. subtilis*. MinD was shown to form larger complexes at the membrane, or, in case of the *E. coli* protein co-polymers with MinC. However, the observed ATPase activities follow Michaelis-Menten kinetic and are not cooperative, indicating that the MinD dimer is the active component.

With the goal of testing binding of isolated MinD to the membrane, we designed a Bio-layer interferometry experiment in which we immobilized liposomes and measured binding of purified MinD proteins. Importantly, without addition of ATP (and ADP present from purification) we observed only very weak MinD binding to the liposomes (Fig. S 8), indicating that MinD membrane binding and dissociation is coupled to the ATP hydrolysis cycle. In presence of ATP, MinD readily binds to the liposomes with high affinity. Members of the ParA/MinD protein family have been

extensively studied and a set of mutations is well characterized that allows generation of strictly monomeric MinD (G12V and K16A), a strictly dimeric MinD variant (D40A) and a membrane binding mutant (I260E) (66 [↗](#)-68 [↗](#)). The most intriguing observation of the binding experiments is that MinD monomers can apparently bind to the liposome membrane *in vitro*. Both monomeric mutants (G12V and K16A) are readily binding to the membrane, but the release of the proteins from the membrane surface differs drastically. While the K16A mutant, unable to bind ATP, has a slow membrane release rate (k_{off} rate of only $2.77 \times 10^{-3} \text{ s}^{-1}$), the G12V monomeric mutant, which can still bind ATP, is released fast from the membrane (k_{off} rate $47.8 \times 10^{-3} \text{ s}^{-1}$). The D40A mutant of MinD, which is thought to be a stable ATP-bound dimer, releases also with very slow kinetics (k_{off} rate $1.76 \times 10^{-3} \text{ s}^{-1}$), indicating that ATP hydrolysis is accelerating release of the MinD proteins from the membrane and that ATP binding itself seems to affect association and dissociation dynamics of MinD. These findings crucially support a reaction diffusion model in which the MinD ATPase hydrolysis cycle is essential for pattern formation of *B. subtilis* MinD. It would have been desirable to study the interaction of MinD with reconstituted MinJ proteins to investigate the influence that MinJ has on membrane interaction of MinD. Unfortunately, we did not succeed in the purification of a full length MinJ protein so far. MinJ contains seven predicted transmembrane helices rendering isolation difficult. Also, the protein was prone to degradation. However, a MinJ mediated stabilization of MinD at the membrane would be comparable to the stabilizing effect that MinE has on MinD oligomers in *E. coli* (93 [↗](#), 94 [↗](#)), and was previously observed in our *in vivo* FRAP studies (60 [↗](#)).

To get an idea of the MinD membrane interaction under *in vivo* conditions, we therefore turned to state-of-the-art single molecule microscopy (SMLM) (95 [↗](#), 96 [↗](#)). We expressed MinD and all MinD point mutants as HaloTag fusions from their native locus in the *min* operon (allelic replacement), similar to our previous work (60 [↗](#)). This ensures native copy number regulation and is a difference to most other studies on Min protein localization and dynamics in *E. coli* or *B. subtilis*.

The locked MinD dimers (D40A mutant) frequently nucleated into larger membrane clusters. This cluster formation is underlined by the drastic increase of the confined/immobile population (54.2%) of D40A in SMLM. This increase in the static population comes to the expense of freely diffusive protein, indicating that the vast majority of MinD D40A is membrane associated. We observed a clear enrichment of these MinD clusters at the cell poles and septa, lending support to the notion that MinD dimers are stabilized/recruited by MinJ. This interpretation is supported by previous data showing that in absence of MinJ, MinD proteins are randomly localized along the membrane in cluster, but not enriched at poles or (in a *minJ* mutant background rare) septa (56 [↗](#)). In line with this idea, the monomeric MinD variants (G12V, K16A) are randomly bound to the membrane surface along the entire cell length. The fact that we find most monomeric MinD proteins under the *in vivo* conditions membrane associated also supports our *in vitro* measurements that MinD can bind in its monomeric form to the membrane in *B. subtilis*. The time-resolved MinD gradient is lost in all mutants, including D40A which should still be recruited by MinJ, suggesting the ATPase cycle and thus MinD cycling is indeed necessary for proper formation of a gradient, as suggested before (60 [↗](#)). Our data are in perfect agreement with recent *in vitro* data collected with the *E. coli* MinD using mass-sensitive particle tracking (MSPT) (77 [↗](#)). MSPT analysis revealed that the *E. coli* MinD forms lateral interactions, leading to higher order oligomers (trimer, tetramer and even higher oligomers). It was speculated that dimerization of *E. coli* MinD at the membrane leads to conformational changes opening an additional interaction surface of MinD for lateral interaction (77 [↗](#)). These lateral MinD interactions were thought to recruit further monomeric MinD from solution to the membrane, thereby forming a nucleation for larger clusters. The MinD cluster formation that we observed in *B. subtilis* using SMLM (60 [↗](#)) are likely *in vivo* evidence for this cooperative membrane binding. It should be noted that the MSPT analysis was not sensitive enough to detect monomer binding to the membrane, but this was explicitly not ruled out (77 [↗](#)). Addition of a second amphipathic helix to the *E. coli* MinD however, was shown to promote monomer binding of *E. coli* MinD to the membrane (65 [↗](#)). Thus, the

difference between the *E. coli* and *B. subtilis* MinD is likely the higher membrane binding capacity of the *Bacillus* protein. The observed cooperativity in membrane binding and thus nucleation, as shown for the *E. coli* MinD *in vitro* (65, 77) and here for the *B. subtilis* MinD *in vitro* and *in vivo*, is required for pattern formation by conferring nonlinearity (60, 97, 98).

In line with these ideas, our SMT data revealed that MinD proteins are found predominantly in three populations with distinct diffusion coefficients. The MinD I260A mutant, impaired in membrane binding, has a largely increased dynamic population, representing the freely diffusive proteins in the cytoplasm. Therefore, we can assign the fastest population of the other MinD variants, including the MinD wild type, with the diffusive, cytosolic fraction and the slower mobile fraction with membrane associated proteins. The confined fraction is therefore likely representing proteins that are either part of larger clusters or interact with other proteins, thereby reducing the dynamicity. Furthermore, this likely indicates that all MinD proteins in a cluster contribute to membrane binding. The two monomeric MinD mutants have a reduced confined fraction in SMT analysis. This global comparison is also supported by a detailed look into the diffusion coefficients of all MinD variants. These variant specific diffusion coefficients can be obtained by analyzing the data shown in Fig. 4 individually (non-simultaneous SQD analysis in SMTracker2 (Tab. 2)). For the D40A mutant all three populations: membrane associated cluster ($D=0.021 \mu\text{m}^2 \text{s}^{-1}$), membrane bound ($D=0.063 \mu\text{m}^2 \text{s}^{-1}$) and cytoplasmic diffusible populations ($D = 0.44 \mu\text{m}^2 \text{s}^{-1}$) display decreased diffusion coefficients compared to wild type (and monomeric MinD variants). The freely diffusible and the membrane associated wild type MinD protein are significantly faster ($D=0.6 \mu\text{m}^2 \text{s}^{-1}$ and $D=0.084 \mu\text{m}^2 \text{s}^{-1}$, respectively). The stable monomeric MinD variants (K16A and G12V) display consistently higher diffusion coefficients, as expected. These data strongly argue for a cytoplasmic pool of MinD proteins that can attach and release from the membrane surface in an ATP depended fashion.

Thus, we conclude that membrane bound MinD dimers have a much stronger tendency to interact with and recruit binding partners (including self-nucleation). This assumption is in good agreement with findings of a recent report that analyzed MinD dynamics *in vivo* using diffraction limited microscopy (99). In this study, a MinD D40A mutant hyper-recruited MinC, leading to cluster formation of MinCD at the membrane. Deletion of MinC restored the MinD gradient formation, indicating that MinC might indeed be part of a MinC/MinD co-polymer, as suggested for the *E. coli* system (100).

Collectively, our data reveal that the MinD ATPase cycle is essential for regulated membrane binding and release, which is crucial for the formation of a sharp MinD gradient at the septum (60). However, in contrast to *E. coli* MinD, monomeric *B. subtilis* MinD can bind the membrane with higher affinity, suggesting that MinD also dimerizes at the membrane surface. Dimer formation is essential for ATPase activity, as indicated by our *in vitro* measurements, and is necessary for efficient membrane detachment. The main characteristics of the *E. coli* MinD with respect to membrane binding, self-interaction leading to nucleation, and membrane detachment are therefore shared in the *B. subtilis* protein. These are the essential requirements for Turing pattern formation by reaction diffusion. The difference, that ATP hydrolysis of the *B. subtilis* MinD does not require a trigger other than membrane binding, makes sense in a system that does not need to oscillate, but rather accumulate and sustain a sharp gradient at the current site of division.

Materials and Methods

Bacterial strains, plasmids and oligonucleotides

Plasmid and strain construction of all relevant constructs is described in the supplementary information. Oligonucleotides, plasmids and strains used in this study are listed in Tab. S 1, Tab. S 2 and Tab. S 3, respectively.

Media and growth conditions

E. coli was grown on lysogeny broth (LB) agar plates containing 10 g L⁻¹ tryptone, 5 g L⁻¹ yeast extract, 10 g L⁻¹ NaCl and 1.5% (w/v) agar at 37°C overnight using appropriate selection (kanamycin 50 µg ml⁻¹, chloramphenicol 35 µg ml⁻¹).

B. subtilis was grown on nutrient agar plates using commercial nutrient broth and 1.5% (w/v) agar at 37°C overnight. To reduce inhibitory effects, antibiotics were only used for transformations and when indicated, since allelic replacement is stable after integration (kanamycin 5 µg ml⁻¹, spectinomycin 100 µg ml⁻¹).

For microscopy, *B. subtilis* was inoculated to an OD₆₀₀ 0.05 from a fresh overnight culture and grown in MD medium - a modified version of Spizizen Minimal Medium (101 [DOI](#)) - at 37°C with aeration in baffled shaking flasks (200 rpm) to OD₆₀₀ 0.5-0.8. MD medium contains 10.7 mg ml⁻¹ K₂HPO₄, 6 mg ml⁻¹ KH₂PO₄, 1 mg ml⁻¹ Na₃ citrate, 20 mg ml⁻¹ glucose, 50 µg ml⁻¹ L-tryptophan, 11 µg ml⁻¹ ferric ammonium citrate, 2.5 mg ml⁻¹ L-aspartate and 0.36 mg ml⁻¹ MgSO₄ and was always supplemented with 1 mg ml⁻¹ casamino acids. Subsequently, cultures were diluted to OD₆₀₀ 0.05 in fresh, pre-warmed MD medium and grown to OD₆₀₀ 0.5 (exponential phase).

Purification of His-MinD and variants

For heterologous expression of His-MinD and its variants, freshly transformed BL21(DE3)/pLysS cells carrying the respective pET-28a expression vector (Tab. S 2) were transferred to LB medium with appropriate selection (kanamycin 50 µg ml⁻¹, chloramphenicol 35 µg ml⁻¹) and grown overnight (37°C, 200 rpm). The next day, fresh LB was inoculated from the overnight culture 1:150 and grown to OD₆₀₀ 0.5 (37°C, 200 rpm) before IPTG (1 mM) was added to induce His-MinD expression. Cells were harvested after 3 h, collected via centrifugation (4°C, 5000 x g for 15 min) and stored at -80°C. For purification, a protocol of *E. coli* MinD purification was adapted from (62 [DOI](#)): the cell pellet was resuspended on ice in lysis buffer (50 mM NaH₂PO₄, 500 mM NaCl and 10 mM imidazole; pH 8.0), supplemented with protease inhibitor cocktail (Roche), 0.2 mM Mg²⁺-ADP and ~250 U mL⁻¹ DNase I (Roche), and subsequently lysed in a pre-cooled (4°C) French pressure cell (Amico; 3 x 1,200 psi). The debris was then removed via centrifugation (10,000 g, 30 min, 4°C) and the supernatant was passed through a 1 ml Ni-NTA column (Protino, Macherey-Nagel) utilizing liquid chromatography (ÄKTApurifier). Here, the resin was washed with 10 ml binding buffer A (50 mM NaH₂PO₄, 500 mM NaCl, 20 mM imidazole; pH 8) before elution buffer B (50 mM NaH₂PO₄, 500 mM NaCl, 250 mM imidazole; pH 8) was added sequentially at concentrations of 5% (10 ml) 10% (20 ml), and finally 100% (10 ml). His-MinD eluate was collected in 0.5 ml fractions. Fractions containing high concentrations of protein (determined by chromatogram analysis, sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting using Penta-His antibody (QIAGEN)) were pooled and concentrated using Amicon filter devices with 10 kDa molecular weight cut-off (Millipore; Merck). Additionally, protein aggregates were removed by pelleting them through centrifugation (10,000 g, 10 min, 4°C).

Next, the sample was purified using size-exclusion chromatography in gel filtration buffer C (50 mM HEPES-KOH, 150 mM KCl, 10% (v/v) glycerol, 0.1 mM EDTA, supplemented with 0.4 mM TCEP and 0.2 mM Mg²⁺-ADP just before use; pH 7.2) through a Superdex200 Increase 10/300 GL column (Cytiva) at a flow rate of 0.75 ml min⁻¹. Fractions containing His-MinD were pooled and either directly used for the respective assays or stored at 4°C for a maximum of one week. Final protein concentration was determined using Bradford assay with bovine serum albumin (BSA) as standards. Protein purity and stability was confirmed via SDS-PAGE using 12% polyacrylamide gels and Western blotting using Penta-His antibody (QIAGEN) (Fig. S 1, Fig. S 5, Fig. S 6, Fig. S 7 and Fig. S 3).

ATP hydrolysis assays

ATP hydrolysis was analyzed in continuous reactions using the EnzChek Phosphate Assay Kit (Thermo Fisher Scientific), according to the manufacturer's manual. Reaction volumes were reduced to 100 μ l and assayed in flatbottom 96-well plates (Greiner-UV-Star 96-well plates). Reactions were measured at 37 °C continuously every minute over a time-course of 3 h in a Tecan Infinite 200 Pro with the Software Tecan i-control v.2.0. If not indicated differently, the reactions contained either 0-16 μ M His-MinD or the respective variant as well as 2 mM Mg^{2+} -ATP and 0.2 mg ml^{-1} liposomes. Liposomes were made from *E. coli* total extract (Avanti) by first drying and then resuspending and rehydrating lipids in gel filtration buffer C (50 mM HEPES-KOH, 150 mM KCl, 10% (v/v) glycerol, 0.1 mM EDTA). Next, the lipids were extruded 40 times through 100 nm polycarbonate membranes (Nuclepore™ Track-Etched Membrane 0.1 μ m, Whatman). Before starting the reactions with 2 mM Mg -ATP, the plate was preincubated for 10 min in order to eliminate phosphate contamination.

Data were first analyzed with Excel (normalized, subtraction of ATP auto hydrolysis and subtraction of no substrate control), a linear regression was used to determine the hydrolysis rate per minute. Data visualization and further analysis was performed using GraphPad Prism version 9.4.0 for Windows, (GraphPad Software).

AlphaFold prediction and structure-based analysis

A structural model of *B. subtilis* MinD was calculated using AlphaFold2 (102 [A](#)) installed on a local computer (Fig. 2 [A](#)), where the structural homology search was performed against the full AlphaFold database as of 11.02.2024 (102 [A](#)).

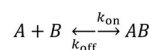
Bio-layer Interferometry

Measurements were performed on the BLItz platform (Sartorius) using Streptavidin (SA) Biosensors (Sartorius, 18-5019). The BLItz device was operated with the Software BLItz Pro (version 1.3.1.3) using the advanced kinetics protocol modified as shown in Table S5 and essentially as described before (103 [A](#)). Beforehand, Liposomes were made from a 33:1 mixture of *E. coli* total extract (Avanti) and DSPE-PEG₍₂₀₀₀₎-biotin (Avanti) by first drying and then resuspending and rehydrating lipids in gel filtration buffer C (50 mM HEPES-KOH, 150 mM KCl, 10% (v/v) glycerol, 0.1 mM EDTA). Next, the lipids were extruded 40 times through 100 nm polycarbonate membranes (Nuclepore™ Track-Etched Membrane 0.1 μ m, Whatman). Biosensors were hydrated for at least 10 min in a 96-well plate in 200 μ l gel filtration buffer C.

As a starting point, unspecific binding was tested. For this, non-biotinylated His-MinD was used to be loaded onto the Streptavidin biosensor. For the binding assays, all measurements were performed in black reaction tubes with 200 μ l gel filtration buffer C or the indicated solution (Tab. S 4). After measuring the initial baseline, biotinylated liposomes were allowed to bind the biosensor. Another baseline was measured in the same tube as before. For association, 200 μ l solutions of His-MinD and variants in different concentrations, respectively, were exposed to the sensor, first without and later with addition of 2 mM Mg^{2+} -ATP (final concentration) just before use. Furthermore, unspecific binding to liposome-saturated sensors was tested using bovine serum albumin (BSA, 16 μ M). For dissociation, the sensor was exposed to buffer again. Each measurement was performed with a fresh biosensor.

Kinetic analysis and fitting were performed by the BLItz Pro Software (version 1.3.1.3). Data was exported and visualized using GraphPad Prism version 9.4.0 for Windows, (GraphPad Software).

The BLItz Pro Software analysis follows a 1:1 binding model, where:



with k_{on} = rate of association or “on-rate” and k_{off} = rate of dissociation or “off-rate”. Formula for fitting the association (a = slope):

$$Y = Y_0 + a (1 - e^{-k_{\text{obs}} \times t})$$

$$k_{\text{obs}} = k_{\text{on}} \times [A(M)] + k_{\text{off}}$$

Formula for fitting the dissociation:

$$Y = Y_0 + a \times e^{-k_{\text{off}} \times t}$$

Resulting in the equilibrium dissociation constant K_D :

$$K_D = \frac{k_{\text{off}}}{k_{\text{on}}}$$

Fluorescence microscopy

Microscopy slide preparation

For epifluorescence imaging, *B. subtilis* cells were mounted on 1.5% MD agarose pads using 1.5 x 1.6 cm “Gene Frames” (Thermo Scientific), and incubated 10 min at 37°C before microscopic analysis.

For SMLM, slides and coverslips were first cleaned by overnight storage in 1 M KOH, carefully rinsed with ddH₂O, and subsequently dried with pressurized air. Next, 1.5% (w/v) low melting agarose (Sigma-Aldrich) was dissolved in MD medium. Medium was sterile filtered (0.2 µm pore size) shortly before being used to remove particles. To produce flat, uniform, and reproducible agarose pads, 1.5 x 1.6 cm “Gene Frames” (Thermo Fisher) were utilized, and pads were allowed to solidify for 1 h at room temperature to be used within the next 3 h.

Staining of HaloTag

For staining, 1 mL of bacterial culture was moved to a 2 mL microcentrifuge tube and incubated with 5 nM (SMLM) or 50 nM (epifluorescence) HaloTag TMR ligand at 37 °C for 10 min. Cells were harvested (4000 g, 3 min, 37 °C) and washed four times in pre-warmed MD medium, before being mounted on the previously described microscopy slides.

Epifluorescence imaging

For strain characterization (**Fig. 2** [B](#)), microscopy images were taken on a Axio Observer 7 microscope (Zeiss) equipped with a Colibri 7 LED light source (Zeiss) and an OrcaR² camera (Hamamatsu) using a Plan-Apochromat 100×/1.4 oil Ph3 objective (Zeiss). TMR fluorescence was visualized with a 90 HE LED filter set (QBP 425/30 + 514/30 + 592/30 + 709/100)(Zeiss) and excited at 555 nm (100% intensity of Colibri 7 LED) for 400 ms. The microscope was equipped with an environmental chamber set to 37 °C. Digital images were acquired with Zen Blue (Zeiss) and analyzed and edited with Fiji ([104](#) [B](#)) ImageJ2 ([105](#) [B](#)).

SMLM imaging

SMLM imaging was performed with an Elyra 7 (Zeiss) inverted microscope equipped with two pco.edge sCMOS 4.2 CL HS cameras (PCO AG), connected through a DuoLink (Zeiss), only one of which was used in this study. Cells were observed through an alpha Plan-Apochromat 63x/1.46 Oil Korr M27 Var2 objective, yielding a final pixel size of 97 nm. During image acquisition, the focus was maintained with the help of a Definite Focus.2 system (Zeiss). Fluorescence was excited with a 561 nm (100 mW) laser, and signals were observed through a multiple beam splitter (405/488/561/641 nm) and laser block filters (405/488/561/641 nm) followed by a Duolink SR QUAD (Zeiss) filter module (secondary beam splitter: LP 560, emission filters: EF BP420-480 + BP495-550).

Cells were illuminated with the 561 nm laser (50% intensity) in TIRF mode (62° angle). For each time lapse series 10000 frames were taken with 20 ms exposure time (~24 ms with transfer time included) and 50% 561 nm intensity laser.

SMLM analysis

For average localization analysis (**Fig. 4** [A](#)), a single-molecule localization table was constructed from a 2D Gaussian fitting in the ZEN 3.0 SR (black) software (Zeiss) with a peak mask size of 9 and a peak intensity-to-noise ratio of 6, and further allowing molecules to overlap. Filtering was used to minimize noise, background, and out-of-focus emitters, discarding events that did not emit within a Point spread function size (PSF) of 100-200 nm and did not display a photon count of 50-600. Further analysis was performed using individual Fiji ([104](#) [B](#)) scripts for the cell outlines and R (4.2.2) scripts in R Studio (2023.12.1+402) for the corresponding localization events ([75](#) [B](#), [76](#) [B](#)). Here, the remaining events were transformed to align the orientation of all cells in which the signals were detected, to be able to summarize all recorded localizations in one normalized cell per strain. The localization frequency is displayed as a heatmap using the gradual viridis scaling from the ggplot2 R package ([106](#) [B](#)), where the highest frequency (normalized frequency 1) is displayed in yellow and the lowest frequency (normalized frequency 0) in blue (**Fig. 4** [B](#)).

For single-molecule tracking, spots were identified with the LoG Detector of TrackMate v6.0.1 ([107](#) [B](#)) implemented in Fiji 1.53 g ([104](#) [B](#)), an estimated diameter of 0.5 μ m, and median filter and sub-pixel localization activated. The signal-to-noise threshold for the identification of the spots was set at 7. To limit the detection of particles to single-molecules, frames belonging to the bleaching phase of TMR were removed individually from the time lapses prior to the identification of spots. Spots were merged into tracks via the “Simple LAP Tracker” of TrackMate, with a maximum linking distance of 500 nm and no frame gaps allowed. Only tracks with a minimum length of 5 frames were used for further analysis, yielding a minimum number of total tracks per sample of 2596.

To identify differences in protein mobility and/or behavior, the resulting tracks were subjected to stationary localization analysis (SLA), mean-squared-displacement (MSD) and square displacement (SQD) analysis in SMTracker 2.0 ([73](#) [B](#)). SLA analysis was performed with a confinement circle radius of 97 nm and a minimum number of 5 steps to be considered static. The average MSD was calculated for four separate time points per strain (exposure of 20 ms - τ = 24, 48, 72 and 96 ms), followed by fitting of the data to a linear equation. The last time point of each track was excluded to avoid track-ending-related artifacts. The cumulative probability distribution of the square displacements (SQD) was used to estimate the diffusion constants and relative fractions of up to three diffusive states. Diffusion constants were determined simultaneously for the compared conditions, therefore allowing for a more direct population fractions comparison. Additionally, the analysis was performed for each strain non-comparatively, yielding individual diffusion coefficients, displayed in **Tab. 2** [B](#).

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Author Contributions

M.B. and H.F. conceived the study, H.F. constructed the strains, performed the in vivo and in vitro experiments incl. microscopy. H.F. and M.B. analyzed the data and wrote the manuscript.

Declaration of Interests

The authors declare no competing interests.

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Reviewer #1 (Public review):

Summary:

In this work, the authors investigate the molecular dynamics of MinD, a component of the *Bacillus subtilis* Min system, in vitro and in vivo. In *Escherichia coli* the Min system is highly dynamic and displays rapid pole-to-pole oscillation whereby a time average minimum of the Min proteins at mid-cell is established. However, in *B. subtilis*, this is not the case, and there is no MinE present. MinD in *B. subtilis* dynamically relocates from the poles to division sites and binds to MinC and MinJ, which mediates its interaction with DivIVA. This paper reports the biochemical characterization of *B. subtilis* MinD in vitro and dynamics of MinD variants in vivo, providing mechanistic insight into the mechanism of dynamic localization.

Strengths:

In the current study, the authors perform a detailed biochemical characterization of the *in vitro* ATPase activity of MinD and demonstrate that rapid hydrolysis is elicited by adding phospholipids. They further show using a collection of substitution mutants of MinD that both monomers and dimers bind to the membrane, and ATP occupancy changes the on and off rates. Identification, quantification, and tracking of discrete Halo-MinD populations were nicely done and showed that mutations in MinD alter dynamic localization, correlating with PL binding on and off rates *in vitro*.

Weaknesses:

While the study shows that MinD in *B. subtilis* utilizes a different (MinE-independent) activation mechanism, it remains to be determined the extent to which MinJ and/or MinC play a role.

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Reviewer #2 (Public review):

Summary:

Feddersen & Bramkamp determined important characteristics of how MinD protein binds/dissociates to/from the membrane, and dimerizes in relation to its ATPase activity. The presented data clearly shows the differences in function of MinD homologs from *B. subtilis* and *E. coli*.

Strengths:

The work presents well-executed experiments that lead to interesting conclusions and a new model of how Min system works during *B. subtilis* mid-cell division. Importantly, this model is supported by *in vitro* characterization of well-chosen mutants in the functional domains of MinD. Outstandingly, most of the *in vitro* data are confirmed by single-molecule localization microscopy.

Weaknesses:

The authors immobilized liposomes, for which they used *E. coli* total lipids, to measure ATPase activity and liposome association and dissociation of *B. subtilis* MinD. For these experiments would be more suitable to use *B. subtilis* total lipids as more biologically relevant data could be gained.

Although the work is in detail and nicely compares the function of *B. subtilis* Min system with *E. coli* Min system, it lacks the comparison of the Min system function in other rod-shaped Gram-positive bacteria. I would suggest including in the Discussion the complexity of other Min systems. Especially, this complexity is seen in other rod-shaped and spore formers such as Clostridial species in which one of these Min systems or both are present, an oscillating *E. coli* Min system type and more static as in *B. subtilis*.

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Reviewer #3 (Public review):

Experimentally, this study provides sufficient data to support the authors' conclusion that MinD dimerization but not ATPase activity is both necessary and sufficient for concentrating it and its binding partner, the division inhibitor MinC, at cell poles. Biochemical data appears to be rigorously acquired and includes proper controls. Although cytological data are

consistent with the authors' model, quantitative information on MinD localization in a statistically relevant set of cells is missing (e.g. Figure 2B).

The study's other major conclusion, as outlined in their discussion, that a reaction-diffusion model explains MinD localization in wild-type cells, is unsubstantiated. If they would like to make this a major conclusion of the final manuscript, they will need to include modeling that takes into account biochemical and cytological data.

From a presentation perspective, the manuscript is challenging to read and will require substantial rewriting and revision prior to publication.

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