Assembly of *Bacillus subtilis* Dynamin into Membrane-Protective Structures in Response to Environmental Stress Is Mediated by Moderate Changes in Dynamics at a Single Molecule Level

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**Abstract**

Dynamin-like proteins are membrane-associated GTPases, conserved in bacteria and in eukaryotes, that can mediate nucleotide-driven membrane deformation or membrane fusion reactions. *Bacillus subtilis*’ DynA has been shown to play an important role in protecting cells against chemicals that induce membrane leakage, and to form an increased number of membrane-associated structures after induction of membrane stress. We have studied the dynamics of DynA at a single molecule level in real time, to investigate how assembly of stress-induced structures is accompanied by changes in molecule dynamics. We show that DynA molecule displacements are best described by the existence of three distinct populations, a static mode, a low-mobility, and a fast-mobile state. Thus, DynA is most likely freely diffusive within the cytosol, moves along the cell membrane with a low mobility, and arrests at division sites or at stress-induced lesions at the membrane. In response to stress-inducing membrane leakage, but not to general stress, DynA molecules become slightly more static, but largely retain their mobility, suggesting that only few molecules are involved in the repair of membrane lesions, while most molecules remain in a dynamic mode scanning for lesions. Our data suggest that even moderate changes in single molecule dynamics can lead to visible changes in protein localization patterns.

**Introduction**

Dynamins are a class of GTPases whose major function lies in the remodeling of biological membranes, most prominently in the mechanical, GTP-driven pinching off of membrane vesicles. Other functions include organelle division and fusion of mitochondrial membranes [Ramachandran and Schmid, 2018]. Bacteria also contain dynamin-like proteins (DLP), whose functions lie in the involvement in bacterial cell division [Dempwolff et al., 2012; Schlimpert et al., 2017] and response to membrane stress [Sawant et al., 2016], likely in the rescaling of holes in the membrane induced by chemical agents or phage evasion. Thus, the overarching scheme of dynamins are membrane fusion and fission [Antonny et al., 2016].

Mechanochemical membrane remodeling involves different domains of dynamins, mediating membrane binding and oligomerization, and GTP hydrolysis-driven...
conformational changes that can generate large membrane rearrangements [Jimah and Hinshaw, 2019]. In general, DLPS consist of a GTPase domain and a stalk domain, and often additional functional domains. The structure of a bacterial DLP has revealed a so-called paddle domain-mediating binding to the lipid bilayer, and a conformational change upon GTP binding, resulting in bending and tabulation of membranes [Low and Lowe, 2006; Low et al., 2009]. Dimerization has also been shown to be important for membrane-binding.

DynA of *Bacillus subtilis* is one of the best studied members of the family of bacterial DLPS. Most bacteria contain two genes encoding for DLPS in an operon, whose products act as a heterotetramer [Liu et al., 2018]. In *B. subtilis*, there is only one gene, *ypbR*, but its corresponding DLP consist of two GTPase domains, with a low affinity to GTP, followed by a stalk and loop region [Praefcke and McMahon, 2004]. Most likely, this gene has arisen through a gene duplication and fusion event.

Even though *B. subtilis* DynA binds to the membrane, it does not contain a transmembrane domain, like other heterotetrameric DLPS. Binding to and fusion of the membrane is accomplished by the two subunits, D1 and D2, which form intrinsic dimers and include the GTPase domain, stalk and loop. D1 might mediate the binding to the membrane, which is independent of nucleotide-binding, and also seems to be responsible for tethering together membranes. For this activity, DynA of *B. subtilis* needs Mg$^{2+}$ to hydrolyze GTP [Burmann et al., 2011]. D2 on the other hand seems to be more of a supporter for fusion and plays only a minor role in binding to the membrane. At the same time, it seems to be necessary for the stability of the binding and cluster formation at the membrane [Guo and Bramkamp, 2019]. DynA might also interact with other proteins via its subdomains D1 and D2, and together with them may lead to membrane fusion [Burmann et al., 2012; Guo and Bramkamp, 2019].

*B. subtilis* DynA has been shown to be present at the division septum, but also at sites along the lateral cell membrane [Dempwolff et al., 2012]. After induction of membrane leakage, DynA foci increase in number at the cell membrane [Sawant et al., 2016]. Importantly, a *dynA* deletion leads to sensitivity of cells towards membrane stress, most pronounced against agents that induce membrane leakage, suggesting that dynamin mediates resealing of holes in the cell membrane [Sawant et al., 2016]. A *dynA* deletion strain also accelerates the cell division phenotype of a *divIB* deletion [Dempwolff et al., 2012], indicating that dynamin may also aid in membrane fusion of the invaginating division septum.

In this work, we wished to further characterize *B. subtilis* dynamin by studying its motion at a single molecule level and to analyze how the assembly of membrane-associated fluorescent foci of a functional DynA-fluorescent protein fusion is brought about by changes of molecular motion. We show that the dynamics of DynA can be best explained by assuming three populations of molecules with distinct diffusion coefficients. Induction of membrane stress and accompanying increase in membrane-associated assemblies are accompanied by only small changes in the size of DynA molecule populations, showing that visible rearrangements of proteins in a bacterial cell can be achieved by only small changes at a single molecule level.

### Results

**A DynA-mVenus Fusion Forms an Increased Number of Foci in Response to Chemical Inducing Membrane Leakage**

We have previously shown that DynA-GFP accumulates at sites of cell division, but can also form visible foci at the cell membrane away from the division site [Dempwolff et al., 2012]. The Bramkamp group has found that addition of nisin, which induces membrane leakage for ions, induces the formation of fluorescent foci at the cell membrane [Sawant et al., 2016]. Because DynA plays a protective role during membrane stress [Sawant et al., 2016], we wished to investigate if other environmental stresses might lead to an accumulation of DynA at the membrane, and treated cells with different chemical and physical stresses. Figure 1a shows that only a minority of exponentially growing cells (26%, Fig. 1a) showed fluorescent foci, predominantly close to mid-cell (septal area) and at the cell poles and that the number of cells with foci was similar during heat and ethanol stress. However, as quantified in Figure 1b, osmotic stress (sorbitol), osmotic and ionic stress (NaCl), and membrane stress (nisin) lead to a large increase in the number of cells with foci, especially under nisin and NaCl conditions. Compared with exponentially growing cells, where 25% contained a single focus and 1% several foci, 63% of nisin-treated cells contained a single focus and 26% several foci; a total of 50% of cells treated with sorbitol contained DynA-mV signals, and 86% of salt-treated cells (Fig. 1b). Following nisin treatment, the ratio of signals at the septum, the cell poles or the lateral membrane changed considerably, from roughly one-third for each position (many places at the
lateral cell membrane defining a “position”) to 70% lateral membrane at the expense of septal and polar localization (Fig. 1b). These findings are in agreement with data from the Bramkamp laboratory showing a strong increase in membrane-associated foci after treatment with nisin [Sawant et al., 2016], and might imply that DynA assembles into visible structures after many kinds of stresses.

Fig. 1. Epifluorescence analysis of DynA-mVenus foci arising in response to different stress conditions. a YFP channels showing unstressed cells with foci mostly found at the septum, heat stressed cells (42°C), or cells after addition of 10% ethanol (EtOH), 1 M sorbitol, 1 M NaCl, 250 µg bacitracin, or 10 µg nisin. Cells were incubated for 30 min for each stress condition. Scale bar, 2 µm. b Total number of counted cells for each condition, numbers of cells with or without foci, which can be also more than one foci per cell, and where they were localized. “Membrane” refers to foci found at the cell membrane except for mid-cell (“septum”) or at the cell poles (“pole”).

|                | unstressed | %     | Heat 42°C | %     | 10% EtOH | %     | 1M Sorbitol | %     | 1M NaCl | %     | 250 µg Bacitracin | %     | 10 µg Nisin | %     |
|----------------|------------|-------|-----------|-------|----------|-------|-------------|-------|---------|-------|------------------|-------|------------|-------|
| cells total    | 302        | 123   | 132       | 54    | 155      | 351   | 391         |       |         |       |                  |       |            |       |
| cells without foci | 222     | 73.61 | 72        | 58.54 | 99       | 75.00 | 27          | 50.00 | 22      | 14.19 | 206               | 58.69 | 46         | 11.76 |
| cells with one focus | 76     | 25.17 | 45        | 36.59 | 27       | 20.45 | 24          | 44.44 | 104     | 67.10 | 138               | 39.32 | 245        | 62.66 |
| cells with more than one focus | 4   | 1.32  | 6         | 4.88  | 6        | 4.55  | 3           | 5.56  | 29      | 18.71 | 7                  | 1.99  | 100        | 25.56 |
| total number of foci | 84  | 57    | 39        | 4.55  | 6        | 4.55  | 3           | 5.56  | 29      | 18.71 | 7                  | 1.99  | 100        | 25.56 |
| pole           | 28        | 33.33 | 18        | 31.58 | 12       | 30.77 | 12          | 40.00 | 74      | 44.58 | 67                  | 43.79 | 75         | 17.32 |
| septum         | 26        | 30.95 | 24        | 42.11 | 16       | 41.03 | 12          | 40.00 | 43      | 25.90 | 26                  | 16.99 | 56         | 12.93 |
| membrane       | 30        | 38.71 | 15        | 26.32 | 11       | 28.21 | 6           | 20.00 | 49      | 29.52 | 60                  | 39.22 | 302        | 69.75 |
DynA Moves as Three Distinct Fractions of Mobility within Cells

Because epifluorescence microscopy can only detect larger, static assemblies of molecules, but not diffusive molecules, we employed single molecule tracking to obtain more quantitative information on changes of DynA mobility. We investigated the dynamics of single DynA-mVenus molecules using “slim-field” illumination and bleaching of most fluorescent proteins until single molecules remain visible that can be efficiently tracked in real time [Reyes-Lamothe et al., 2010; Kleine Borgmann et al., 2013; Schibany et al., 2018]. We have previously shown that this approach leaves cells physiologically largely intact and harms them only to a small degree [Dersch et al., 2020; El Najjar et al., 2020]. We obtained an average track length of about 8 steps and used tracks of 5 steps and longer for the analyses of diffusion constants and possible existence of several distinct populations of molecules using squared displacement analysis (SQD). Figure 2 shows jump distance (JD) evaluation of SQD data, indicating that the observed step size distributions can be best explained assuming 3 distinct populations (Fig. 2a), leaving almost no residuals when comparing observed data and modeled data (Fig. 2b). When only 2 populations are used (Fig. 2c), observed JDs cannot be fully covered, and residuals remain in panel d, where the blue line deviates visibly from the dotted red line representing the modelled data. Although we do not want to overinterpret our data, we will continue assuming three diffusive populations, and will return to the idea of the existence of only two populations in the discussion section. For three population fits, we obtained the following diffusion constants (D) and population sizes for DynA molecules in unstressed cells, using SMTracker 2.0: 15% of the molecules had a D of 0.015 µm² s⁻¹, about 50% showed a D of 0.122 µm² s⁻¹, and the fastest population of about 35% showed a D of about 0.9 µm² s⁻¹ (Fig. 3a and b). The later D is characteristic of large cytosolic proteins [Schenk et al., 2017; Rosch et al., 2018], while that of 0.015 µm² s⁻¹ reflects a rather static molecule, bound to a much larger structure, such as a ribosome [Sanamrad et al., 2014]. Thus, 15% of DynA molecules appear to be statically positioned within a larger subcellular structure, while 35% are freely diffusive. We will come back to this point later, when we consider the spatial distribution of static and diffusive molecules. Taking into account the findings that DynA is involved in the cell division process and is frequently observed at the septal area (see also Fig. 1a), a fraction of the static DynA molecules is likely bound to the Z ring containing a variety of cell division proteins. If DynA indeed diffuses as three distinct fractions, the medium-mobile fraction might be molecules attached to the membrane, which would be expected to diffuse somewhat slower than cytosolic proteins, or molecules in a (pre) complex of an intermediate size. With the setup used, we cannot determine if molecules move along the membrane or within the cytosol, so the idea of an intermediate, membrane-associated DynA fractions remains a hypothesis.

Fig. 2. Jump distance analysis (JD) of DynA-mV showing the distribution of particle displacements in a fixed time interval, plotted in a histogram (a + c + e–j). The quantile-quantile plots visualize the goodness of the fits, by comparing difference (blue line) between modelled (dashed line) and observed data (b + d). a Unstressed triple fit. b Quantile-quantile plot of the unstressed triple fit. c Unstressed double fit. d Quantile-quantile plot of the unstressed double fit. Because the data imply a triple fit is the better option, the following conditions were analyzed with a triple fit: 42°C (e), 10% ethanol (f), 1 M sorbitol (g), 1 M NaCl (i), 250 µg bacitracin (j), 10 µg nisin (k). h, i Double fits of 1 M sorbitol treatment (h) of 10 µg nisin (l). m–n Examples of trajectories from cells expressing DynA-mV during exponential growth (m) or after addition of nisin (n). (For figure see next page.)
molecules became somewhat more mobile; however, as for the other stress conditions, this effect was not dramatic. Conversely, after the addition of nisin, the size of the static fraction, assumed to be DynA molecules engaged in cell division or in repair of membrane damage, almost doubled, while the number of highly mobile molecules strongly decreased (Fig. 3a). These data support the idea of a specific nisin-induced effect on DynA dynamics and suggest that changes observed by the other stress conditions are likely more unspecific. However, although DynA provides protection for the cell against nisin treatment, overall changes in the whole DynA molecule population are not profound, at least not as large as we would have expected. Only about 13% of all molecules move from a more mobile into a static mode, which we assume is the active form of DynA.

Stress Conditions Lead to Visible Changes in DynA Localization and Mobility

In order to further investigate changes in the localization and mobility of DynA, we generated heat maps, in which tracks from all cells are projected into a medium-sized cell

Fig. 3. Analysis of the dynamics of DynA-mVenus under different stress conditions. For the determination of the diffusion coefficients (D) and the fraction sizes, SQD analyses with simultaneous fits for all conditions were used. This leads to the determination of a single value for D that fits best to all conditions, in order to allow for a better comparison of changes in population sizes. a The bubble plot shows the size of the fraction where each bubble is proportional to the area of its corresponding diffusion coefficients. b In the table, population 1 (“pop 1”) refers to D1, and other sizes accordingly. Note that standard errors refer to fitting errors, not to differences between the biological triplicates, whose data were pooled for the analyses.
Fig. 4. Localization of DynA-mVenus tracks under different stress conditions. All tracks of one condition were projected into a standardized/medium size cell of 1 × 3 µm. From white, via yellow to red is the low to high probability of distribution of the tracks. Panels a, c, e, g, i, k, m show heat maps without mirroring; panels b, d, f, h, j, l, n show double-mirrored heat maps. Note that heat maps are not adjusted to each other.

a, b Unstressed. c, d 42°C. e, f 10% EtOH. g, h 1 M sorbitol. i, j 1 M NaCl. k, l 250 µg bacitracin. m, n 10 µg nisin.
and are displayed for frequent or infrequent presence. Figure 4a shows a slight tendency for DynA to be present close to the cell membrane (Fig. 4b is a twofold mirrored heat map), but even the frequent foci observed at the division site (Fig. 1a) do not shift this preference to the cell center. These data suggest that DynA molecules are distributed throughout the cell, i.e. within the cytosol and at the membrane. Heat and sorbitol stress induced an accumulation of DynA at the subpolar regions (Fig. 4c/d, and g/h), which might be due to some molecule aggregation due to un- or misfolding during heat or osmotic shock. Note that heat maps were not normalized to each other because of the strong subpolar signals observed after heat and osmotic stress induction. Heat maps of ethanol, sodium chloride, and bacitracin stress did not induce any clear changes (Fig. 4e/f, i/j, and k/l). Conversely, DynA molecules showed a higher preference towards membrane-proximal accumulation following nisin treatment (Fig. 4m/n).

We also generated “confinement maps,” in which molecules showing little displacement for an extended time (confined motion) and freely diffusing molecules are displayed in a standardized cell. This analysis must not be confused with SQD analyses, because molecules with a low diffusion constant do not necessarily represent those showing confined motion for some time, although these two parameters will strongly overlap, and the analysis does not distinguish between e.g., populations of medium or of high mobility. For confinement maps, we used a radius of 120 nm (about three times the localization error in the setup) and defined confinement as molecules remaining within this radius for at least 8 time intervals. Figure 5 shows that during exponential growth (Fig. 5a), confined motion (red tracks) occurred throughout the cell, and frequently close to the cell membrane. The distribution of tracks along the length of cells (long axis, or x axis) is shown in Figure 5b, left panel, distribution along the width (short/y axis) in Figure 5h, right panel. Sorbitol and heat stress induced subpolar areas of increased confined motion (best seen in Fig. 5i and k, long axis), possibly reflecting places of enhanced protein aggregation. For ethanol, sodium chloride and bacitracin treatment, no clear changes were observed. Nisin treatment induced the strongest change in spatial dynamics, in that confined motion for some time, although these two parameters leads to two distinguish populations (Fig. 6b). The one-component fit does not include the whole data, while the two-component fit does. While τ1 is with 0.18 s similar to the unstressed one, τ2 increased strongly, to 0.35 s. This longer dwelling event occurred for roughly half of the molecules (48 ± 3.7%). Thus, we can assume that under nisin stress, there is a population which has a shorter average dwelling time (τ1) similar to unstressed cells, while the long dwelling events (τ2) refer to DynA molecules in the membrane that repair nisin-induced damage. Of note, the static population of molecules seen with SQD analyses of DynA, which almost doubled under nisin stress, will largely overlap with the population of molecules showing long dwelling events, but is not necessarily identical. Likewise, the popula-

**Fig. 5.** Confinement maps of DynA under different stress conditions. To visualize confined motion of DynA molecules, a radius of 120 nm was set in a standardized cell of 1 × 3 µm. A minimum of 8 steps was taken to indicate confined motion, shown in red. Freely diffusive tracks are shown in blue, mixed behavior between mobile and confined motion in green. Stress conditions as stated above the panels. a Unstressed. b 42°C. c 10% EtOH. d 1 M sorbitol. e 1 M NaCl. f 250 µg bacitracin. g 10 µg nisin. h–n Histograms of the distribution along the long axis (left panels) or short axis (right panels) of confined tracks, conditions used are indicated above the panels.

(For figure see next page.)
Molecule Dynamics Changes and Protein Localization Patterns

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Fig. 6. Nisin stress leads to longer dwelling events. Histograms show empirical cumulative density function (ecdf) of the length of time of DynA molecules reside in a 120-nm circle for a minimum of 8 time points, shown for the unstressed condition (a), after addition of 10 µg/mL nisin for 30 min (b), or of 250 µg/mL of bacitracin for 30 min (c). τ describes the dwelling event for a one-component fit in green and τ_1 and τ_2 for a two-component fit in red. The average duration for the dwelling event is shown in seconds, while the percentage states the corresponding population size. Errors reflect deviations from the fitted exponential decay curves. Significance values are derived from the t test.

Thus, although a direct comparison of the SQD data with the dwell times cannot be made, it can be said that with both tools we are able to detect static DynA molecules. In both models, under nisin stress, DynA becomes more static, and a clear difference in its single molecule dynamics can be seen. While with SQD analysis, a transition to a more static behavior under bacitracin stress was not apparent, the change in the dwelling events suggests that also under this condition DynA is recruited to the membrane and induces membrane repair assemblies.

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Molecule Dynamics Changes and Protein Localization Patterns

Discussion

Bacterial cells have traditionally been regarded as organisms whose protein dynamics are largely governed by free Brownian diffusion of enzymes. While this may be true for a majority of cellular proteins, many proteins show assembly at distinct subcellular sites at all times, or even at specific times during the cell cycle [Shapiro and Losick, 2000]. It has also been found that diffusion can be anomalous within the bacterial cytoplasm, and its magnitude is affected by metabolic activity of the cell [Parry et al., 2014]. Thus, mobility in non-compartmentalized bacterial cells is non-trivial, and it is still unclear for most proteins complexes in which fashion they are assembled. Diffusion capture has been described for some processes, but other modes appear to be possible.

In our work, we wished to address the question of how molecule dynamics change for a protein known to form assemblies, based on fluorescence microscopy, at a given subcellular site, at a single molecule level, in order to monitor and describe changes between diffusive and bound molecules. We chose DynA from *B. subtilis*, a soluble protein that can associate with the cell membrane via a transmembrane-like dimeric subunit, D1. DynA was shown to assemble at the septum in a subset of cells within a growing population, colocalizing with FtsZ [Dempwolff et al., 2012]. Based on genetic interactions, it was suggested that DynA plays a role in cell division, possible at the last step of fusion of the invaginating cell membrane. Increased membrane binding of DynA was inferred from wide field microscopy using a fluorescent protein fusion of DynA, when cells were treated with an agent inducing membrane leakage, lantibiotic nisin, a compound produced by lactic acid bacteria, with a broad antibacterial spectrum amongst Gram-positive bacteria. In order to obtain more insight into the dynamics of DynA assembly at the cell membrane, we used single molecule tracking, which not only localizes statically bound molecules, but also freely diffusing, mobile molecules. We found that DynA diffusion can be explained by assuming two distinct populations, and to an even better degree by assuming three populations. For a two-population model, one would assume a freely diffusing part and a statically, membrane-engaged part of molecules. We found minor changes in single molecule dynamics using two or three population models for general stress conditions, but considerable changes following nisin treatment. For the purpose of this study, we chose a model with three populations, which might be a better reflection of molecule dynamics within cells, keeping in mind that qualitatively, changes were similar regardless of whether two or three populations were assumed.

Nisin treatment induces or enhances the recruitment of DynA molecules to the cell membrane, and the formation of fluorescent foci as seen by observing a functional fluorescent protein fusion to DynA [Sawant et al., 2016] (Fig. 1). Rather than about 25% of cells showing a single DynA-mVenus focus at the cell membrane during exponential growth, about 80% of cells contain mostly several foci following nisin treatment. We show that this observation made by wide-field fluorescence microscopy involves only about 13% of the molecules, which change from being mobile (regardless of whether one or two mobile populations are assumed) to being static, using single molecule tracking. Accumulation of molecules moving in a confined manner occurred close to the cell membrane, as shown by confinement maps. Considerably longer dwell times that were observed between exponentially growing cells and nisin-treated cells also underline that DynA becomes more static, by assembling in membrane repair complexes.

Using general stress factors such as heat, ethanol, osmotic, and salt stress, we found a visual redistribution of molecules, in that especially after heat and osmotic stress, more molecules of DynA were found to cluster close to the cell poles. We interpret these findings to indicate that some molecules would denature and thus aggregate at the cell poles, possibly soon after translation, which occurs predominantly at the poles [Lewis et al., 2000; Mascarenhas et al., 2001]. However, we did not find any notable changes at a single molecule level after general and membrane stress, different from treatment with nisin. An argument can be made that also Bacitracin treatment leads to significant changes of molecule dynamics, which can be seen for the confined motion, and increased average dwell times that were observed. Both nisin and bacitracin are antibiotics that bind to and affect the cycle of lipid II during cell wall synthesis, and nisin induces membrane leakage [El Jastimi et al., 1999]. These findings suggest that DynA is recruited to membrane damage if there is a specific leakage, e.g. through interference with lipid II components.

The data suggest that for repair of membrane leakage or generally membrane fusion, a minority of DynA molecules becomes statically involved/assemble at the membrane. The remaining molecules continue to be mobile, likely scanning the cell membrane for lesions. Our work shows that assembly of a protein at the cell membrane (in order to mediate membrane integrity) involves a small
exchange in molecule dynamics and thus an induced diffusion/capture mechanism. We speculate that a large majority of protein assembly mechanisms involve diffusion/capture rather than specific membrane integration or proteolysis or other targeted mechanisms.

Materials and Methods

Bacterial Strains and Growth Conditions

\textit{B. subtilis} strain BG214 (also termed YB886) is a derive of the strain 168. It is auxotrophic for tryptophan (\textit{trpC2}) and methionine (\textit{metB5}); therefore, minimal media were supplemented with 50 mg/mL tryptophan and methionine, respectively [Yasbin et al., 1980]. For cloning, the \textit{Escherichia coli} strain DH5a was used. Both strains were cultivated in Luria-Bertani medium, whereas it was used for \textit{B. subtilis} only for overnight cultures. \textit{E. coli} was grown at 37°C, while \textit{B. subtilis} was cultivated at 250 rpm and 30°C. For epi-fluorescence and single-molecule tracking, \textit{B. subtilis} was grown in \textit{S7} \textit{50} minimal medium (1% [w/v] fructose, 0.1% [w/v] glutamate, 0.004% [w/v] casamino acids) [Jaacks et al., 1989] with the same temperature and speed. For the determination of the growth rate, it was measured with an optical density at 600 nm (OD \textsubscript{600}). An OD \textsubscript{600} of 0.8 was used for microscopy. Selection of the strains was accomplished by using the antibiotics ampicillin (100 µg/mL) for \textit{E. coli} and chloramphenicol (5 µg/mL) for \textit{B. subtilis}. In BG214 cells the xylose promoter was induced by adding 0.1% (w/v) xylose to the cells.

Strain Construction

BG214 \textit{DynA}_{mVenus} at the original locus was constructed by firstly creating the plasmid in \textit{E. coli} DH5a. For this, the plasmid pSG1164 (ECI55) with a \textit{mVenus} fluorophore was used. The last 500 bp of the 3'-end of DynA were cloned in front of the fluorophore. The cutting enzymes EcoRI and ApaI were used with the forward primer GCTAGAATTCGACAACAGGCTTTA and the reverse primer GCATGGGGCCATTTTTATGGTATTGTCTG, where the stop codon was removed. After transformation of this plasmid in \textit{E. coli}, the plasmid was checked via sequencing and \textit{B. subtilis} BG214 competent for DNA uptake, an overnight culture of this strain 168. It is auxotrophic for tryptophan (\textit{trpC2}) and methionine (\textit{metB5}); therefore, minimal media were supplemented with 50 mg/mL tryptophan and methionine, respectively [Yasbin et al., 1980]. For cloning, the \textit{Escherichia coli} strain DH5a was used. Both strains were cultivated in Luria-Bertani medium, whereas it was used for \textit{B. subtilis} only for overnight cultures. \textit{E. coli} was grown at 37°C, while \textit{B. subtilis} was cultivated at 250 rpm and 30°C. For epi-fluorescence and single-molecule tracking, \textit{B. subtilis} was grown in \textit{S7} \textit{50} minimal medium (1% [w/v] fructose, 0.1% [w/v] glutamate, 0.004% [w/v] casamino acids) [Jaacks et al., 1989] with the same temperature and speed. For the determination of the growth rate, it was measured with an optical density at 600 nm (OD \textsubscript{600}). An OD \textsubscript{600} of 0.8 was used for microscopy. Selection of the strains was accomplished by using the antibiotics ampicillin (100 µg/mL) for \textit{E. coli} and chloramphenicol (5 µg/mL) for \textit{B. subtilis}. In BG214 cells the xylose promoter was induced by adding 0.1% (w/v) xylose to the cells.

Preparation of BG214 \textit{DynA}_{mVenus} for Microscopy

\textit{B. subtilis} BG214 DynA-mV cells were grown in \textit{S7} \textit{50} minimal medium at 30°C under shaking conditions to an OD \textsubscript{600} of 0.8. Afterwards, the cells were stressed with 1 M sorbitol, 1 M NaCl, 10% EtOH, 250 µg bacitracin or 10 µg nisin for 30 min at 42°C in a shaking bath for 30 min. Cells were spotted on coverslips (25 mm, Marienfeld) and covered with a 1% agarose pad (w/v), made of \textit{S7} \textit{50} medium and a smaller coverslip (12 mm, Marienfeld).

Epifluorescence Microscopy

A Zeiss Axio observer Z1 microscope with a Cascade II:512 Photometrics camera was used for imaging. The magnification of the objective was x100, while the magnification of the optivar was x1.6. The total magnification was x160. The camera chip was 512 × 512 with a pixel size of 16 µm × 16 µm. Images were acquired using Metamorph, and acquisition was done with an exposure time of 3,000 ms using a YFP filter.

Single-Molecule Tracking, Data Acquisition, and Analysis

Imaging was performed with a Nikon Eclipse Ti microscope equipped with a high numerical aperture objective (CFI Apochromat TIRF 100X.C Oil, NA 1.49), an EM-CCD camera (ImagEM.X2, Hamamatsu) and a YFP filter set (BrightLine 500/24, Beamsplitter 520 and BrightLine 542/27). \textit{mVenus} fluorophores were excited by the central part of a laser beam (TOPTICA Beam Smart, 515 nm, max. power 100 mW) with a laser intensity of 150 W cm\textsuperscript{-2}. Each movie consists of 3,000 frames and was recorded with an exposure time of 20 ms, using Nikon NIS-Elements BR.

First, the videos were cut with Fiji (ImageJ) [Schindelin et al., 2012] and the last 2,000 frames were used. Afterwards, the cell meshes were set with Oufiti [Paintdákhi et al., 2016]. For particle detection, U-track [Jaqaman et al., 2008], MATLAB software, was used. Here, the minimal length of tracks was set to 5 and to link to points, no gaps for the particle detection was allowed. Data were analyzed using software SMTTracker 2.0 [Oviedo-Bocanegra et al., 2021]. In this program, the Stationary Localization Analysis panel was used for dwell time analysis and heat maps, and the Square Displacement Analysis (SQA) panel for calculation of diffusion constants are population sizes and confinement maps. SQA uses the squared, 2-dimensional radial displacement of molecules, generating a cumulative distribution function (CDF) of squared. For a given number of diffusive states, the algorithm implemented in SQA performs a simultaneous nonlinear least square fit to the experimental CDFs. SMTTracker estimates the most approximate number of diffusive groups found in the experimental data using the Bayesian information criterion, which also help to avoid overfitting of data. To obtain the uncertainty on the estimation of the fitting parameters, the main dataset is split into two subsets, train and test. Fitting estimation is run using only the training subset and evaluating onset of the test data. The training subset is methodologically split into 10 independent folds without any common data point, and the fitting is performed in each of these. Final estimation of the parameters is obtained after determining the mean, and the error is the sum of the standard error for the mean and the 95% confidence intervals obtained from the fitting procedure. Normalization is done according to standard procedures; sizes of bins in histograms were adjusted to yield the visually best representation of data.

Statement of Ethics

An ethics statement was not required for this study type, no human or animal subjects or materials were used.
Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

L.S. performed all experiments, evaluated data, generated all figures, and helped writing the manuscript. P.L.G. conceived of the study, helped evaluate data, and wrote the manuscript.

Data Availability Statement

All data generated or analyzed during this study are included in this article. Raw data can be requested from and further enquiries can be directed to the corresponding author.

Conflict of Interest Statement

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