Dependence of diffusion in *Escherichia coli* cytoplasm on protein size, environmental conditions and cell growth

N. Bellotto¹, J. Agudo-Canalejo^{2,†}, R. Colin^{1,†}, R. Golestanian^{2,3,+}, G. Malengo^{1,+}, V. Sourjik^{1,+}

¹Max Planck Institute for Terrestrial Microbiology and LOEWE Center for Synthetic Microbiology (SYNMIKRO), Karl-von-Frisch-Strasse 16, D-35043 Marburg, Germany

²Max Planck Institute for Dynamics and Self-Organization, Am Faßberg 17, D-37077 Göttingen, Germany

³Rudolf Peierls Centre for Theoretical Physics, University of Oxford, Oxford OX1 3PU, United Kingdom

[†]These authors equally contributed to this work

⁺Corresponding authors: ramin.golestanian@ds.mpg.de, gabriele.malengo@synmikro.mpi-marburg.mpg.de, victor.sourjik@synmikro.mpi-marburg.mpg.de

Abstract

Inside prokaryotic cells, passive translational diffusion typically limits the rates with which cytoplasmic proteins can reach their locations and interact with partners. Diffusion is thus fundamental to most cellular processes, but the understanding of protein mobility in the highly crowded and non-homogeneous environment of a bacterial cell is still limited. Here we investigated the mobility of a large set of proteins in the cytoplasm of *Escherichia coli*, by employing fluorescence correlation spectroscopy (FCS) combined with simulations and theoretical modeling. We conclude that cytoplasmic protein mobility could be well described by Brownian diffusion in the confined geometry of the bacterial cell and at high viscosity imposed by macromolecular crowding. The size dependence of protein diffusion is well consistent with the Stokes-Einstein relation for small fusion proteins when taking into account their shape, but larger proteins diffuse slower than expected from this relation. Pronounced subdiffusion and hindered mobility is observed for proteins with extensive interactions within the cytoplasm. Finally, we show that cytoplasmic viscosity has a temperature dependence comparable to that of water, and that protein mobility is increased by biosynthetic activity and cell growth.

Introduction

Diffusion of molecules is important for the function of any cellular system, setting the upper limit for mobility of proteins and other (macro)molecules and for the rates of many biochemical reactions that rely on random encounters between molecules (Schavemaker, Boersma, and Poolman 2018). Although the fundamental physics of diffusion in dilute aqueous solutions is well understood and mathematically described (Einstein 1906; Langevin 1908; Perrin 1909), diffusion in a cellular environment may be quite different (Schavemaker, Boersma, and Poolman 2018; Mika and Poolman 2011). The concentration of macromolecules in the bacterial cytoplasm, primarily proteins but also ribonucleic acids (RNAs), is extremely high. For *Escherichia coli*, it is around 300 mg/mL, which corresponds to a volume fraction of 25-30% (Cayley et al. 1991; Zimmerman and Trach 1991). Such macromolecular crowding could hinder free diffusion and influence kinetics of protein association and of gene expression (Klumpp et al. 2013; Tabaka et al. 2014; van den Berg, Boersma, and Poolman 2017). The effects of crowding on protein diffusion have been demonstrated both *in vitro* and *in vivo* (Dix and Verkman 2008; Rivas and Minton 2016). Compared to water, the diffusion of a free green fluorescent protein (GFP) was reported to be three to four times slower in the eukaryotic cytoplasm (Swaminathan, Hoang, and Verkman 1997) and up to ten times slower in the bacterial cytoplasm (Elowitz Michael et al. 1999; Nenninger, Mastroianni, and Mullineaux 2010; Mika et al. 2010; Kumar, Mommer, and Sourjik 2010).

In addition to the high density of macromolecules, the diversity in the size and chemical properties of the solutes makes the cytoplasmic environment highly inhomogeneous (Luby-Phelps 1999; Spitzer and Poolman 2013). How much the diffusion of a particular molecule is affected by macromolecular crowding might thus depend on the size (Muramatsu and Minton 1988) and shape of the molecule (Balbo et al. 2013) as well as on the nature of the crowders (Banks and Fradin

2005; Goins, Sanabria, and Waxham 2008). The effects of crowding observed in living cells appear to be even more complex, varying not only with the diffusing particle but also with the physiological state of the cell (Parry et al. 2014; Joyner et al. 2016) and the local cellular environment (Konopka et al. 2006; Persson, Ambati, and Brandman 2020). Moreover, non-trivial effects on diffusion arise due to reversible assembly and disassembly of the diffusing protein complexes (Agudo-Canalejo, Illien, and Golestanian 2020), and possibly also due to the active enhancement of enzyme diffusion by catalytic reactions (Golestanian 2015; Agudo-Canalejo et al. 2018; Zhang and Hess 2019).

The dependence of the diffusion coefficient (D) of a protein in the cytoplasm on its size might thus not necessarily follow the Stokes-Einstein (also called Stokes-Einstein-Sutherland-Smoluchowski) relation that is valid in dilute solutions, $D \propto T/(\eta R)$ (Einstein 1906), where T is absolute temperature in Kelvin, η is the viscosity of the medium and R is the hydrodynamic radius of the particle. For globular proteins, R is given by the radius of gyration (Tyn and Gusek 1990) and depends on the molecular mass (MM) as $R \propto MM^\beta$, where the exponent β would be 1/3 for perfectly compact and globular proteins but is in practice within the range of 0.35-0.43 for typical proteins, reflecting the fractal nature of the spatial distribution of protein mass (Smilgies and Foltastognew 2015; Enright and Leitner 2005). Several studies of protein diffusion in the cytoplasm of *E. coli* have yielded different dependencies on the molecular mass, with an average $\beta \sim 0.7$ estimated based on the data pooled from multiple studies (Nenninger, Mastroianni, and Mullineaux 2010; Kumar, Mommer, and Sourjik 2010; Mika and Poolman 2011; Kalwarczyk, Tabaka, and Holyst 2012), and thus substantially steeper than predicted by the Stokes-Einstein relation. Similar exponent of ~ 0.7 was observed for a limited set of differently-sized proteins (Mika et al. 2010; Stracy et al. 2021). However, neither of these studies took explicitly into account the non-

globularity of the used fluorescent constructs, where two or more proteins are typically connected by flexible linkers. For such multidomain proteins, shape fluctuations and hydrodynamic interactions between the different domains can have a sizeable effect on the effective diffusion coefficient of the whole protein (Agudo-Canalejo and Golestanian 2020).

Besides macromolecular crowding, the translational diffusion of cytoplasmic proteins is also influenced by intracellular structures, such as cytoskeletal filaments (Sabri et al. 2020), and by (transient) binding to other macromolecules (Guigas and Weiss 2008; Saxton 2007). Both these factors can not only reduce protein mobility but also lead to the anomalous subdiffusive behavior, where the mean square displacement (MSD) of diffusing particles does not scale linearly with time, as for Brownian diffusion in dilute solutions, but rather follows $MSD \propto t^\alpha$ with the anomalous diffusion exponent α being <1 (Saxton 1996; Etoc et al. 2018). Subdiffusion is commonly observed in eukaryotes, particularly at longer spatial scales, primarily due to the obstruction by the cytoskeletal filaments (Di Rienzo et al. 2014; Sabri et al. 2020). Mobility of mRNA molecules in the bacterial cytoplasm is also subdiffusive (Golding and Cox 2004; Lampo et al. 2017). In contrast, the diffusion of several tested cytoplasmic bacterial proteins was apparently Brownian (Bakshi, Bratton, and Weisshaar 2011; English et al. 2011).

Even for the same protein, e.g. GFP or its spectral variants, estimates of the diffusion coefficient in the cytoplasm obtained in different studies vary widely (Schavemaker, Boersma, and Poolman 2018), which could be in part due to differences in methodologies. Most early studies in bacteria relied on fluorescence recovery after photobleaching (FRAP), where diffusion is quantified from the recovery of fluorescence in a region of the cell bleached by a high intensity laser (Loren et al. 2015). These measurements provided values of diffusion coefficient for GFP ranging from $3 \mu\text{m}^2\text{s}^{-1}$ to $14 \mu\text{m}^2\text{s}^{-1}$ (Elowitz Michael et al. 1999; Mullineaux et al. 2006; Konopka et al. 2009; Kumar,

Mommer, and Sourjik 2010; Mika et al. 2010; Nenninger, Mastroianni, and Mullineaux 2010; Schavemaker, Smigiel, and Poolman 2017). More recently, single-particle tracking (SPT), where diffusion is measured by following the trajectories of single fluorescent molecules over time (Kapanidis, Uphoff, and Stracy 2018), became increasingly used. Lastly, diffusion can also be studied *in vivo* using fluorescence correlation spectroscopy (FCS) (Cluzel, Surette, and Leibler 2000), which measures the time required by a fluorescent molecule to cross the observation volume of a confocal microscope (Elson 2011). SPT and FCS measure protein mobility locally within the cell, with FCS having also a significantly better temporal resolution than FRAP and SPT. Both methods provided higher but still varying values of D_{GFP} , from $8 \mu\text{m}^2\text{s}^{-1}$ up to $18 \mu\text{m}^2\text{s}^{-1}$ (Meacci et al. 2006; English et al. 2011; Sanamrad et al. 2014; Diepold et al. 2017; Rocha et al. 2019).

Protein mobility also depends on the environmental and cellular conditions that affect the structure of the cytoplasm (Schavemaker, Boersma, and Poolman 2018). Previously studied examples include effects of high osmolarity that increases macromolecular crowding and creates diffusion barriers (Konopka et al. 2006; Konopka et al. 2009; Liu et al. 2019) and of the energy-dependent fluidization of the cytoplasm to enable diffusion of large particles (Parry et al. 2014). Furthermore, the surface charge of cytoplasmic proteins has been shown to have a dramatic effect on their mobility (Schavemaker, Smigiel, and Poolman 2017).

Variations between values of diffusion coefficients observed even for the same model organism in different studies, each investigating only a limited number of protein probes, using different strains, growth conditions and measurement techniques, hampered drawing general conclusions about the effective viscosity of bacterial cytoplasm and its dependence on the protein size. Here, we address these limitations by systematically analyzing the mobility of a large number of differently-sized cytoplasmic fluorescent protein constructs under standardized conditions by FCS.

We further combined experiments with Brownian dynamics simulations and theoretical modeling of diffusion to correct for effects of confined cell geometry. For the majority of studied constructs, we observe a uniform dependence of the diffusion coefficient on the protein size, with a pronounced upper limit on diffusion at a given molecular mass. When corrected for the confinement due to the bacterial cell geometry, the diffusion of these constructs was nearly Brownian. Moreover, part of the deviation of the mass-dependence of their diffusion coefficients from the Stokes-Einstein relation might be explained by the specific shape of the fusion proteins. An aberrantly slow and anomalous diffusion of several protein constructs was apparently due to their strong interactions with other cellular proteins and protein complexes, and disruption of these interactions restored a Brownian diffusion close to the upper limit expected for their mass. We further investigated the dependence of protein mobility on environmental temperature, on exposure to antibiotics and on cellular growth, and we tested the mobility of proteins that are non-native to *E. coli*. Finally, we show that under the same experimental conditions FCS and FRAP measurements yield similar values of diffusion coefficients, suggesting that, in contrast to eukaryotic cells (Di Rienzo et al. 2014), no pronounced dependence of protein mobility on spatial scale could be observed in the bacterial cytoplasm.

Results

Dependence of cytoplasmic protein mobility on molecular mass measured by FCS

For our analysis of cytoplasmic protein mobility, we generated a plasmid-encoded library of 31 cytoplasmic proteins (Figure 1 – supplementary table 1 and supplementary table 2) of *E. coli* fused

to superfolder GFP (sfGFP) (Pédélec et al. 2006). We selected monomeric proteins with known and roughly globular protein structure, to avoid effects on mobility due to multimerization or irregular shape of proteins. These proteins belong to different cellular pathways, but DNA-binding proteins were excluded. The expected size and stability of each construct was verified by gel electrophoresis and immunoblotting (Figure 1 – figure supplement 1). Only one of the constructs, ThpR-sfGFP, showed >20% degradation to free sfGFP, and it was therefore excluded from further analyses. This was also the sole construct with an atypically high isoelectric point (pI), and all remaining constructs have pI ranging from 5.1 to 6.2, as common for cytoplasmic proteins (Schwartz, Ting, and King 2001). We further imaged the distribution of fusion proteins in the cytoplasm. Except for RihA-sfGFP and NagD-sfGFP that were subsequently excluded, all other constructs showed uniform localization (Figure 1A).

The mobility of the remaining 28 fusion constructs and of free sfGFP was investigated in living *E. coli* cells by FCS (see Materials and Methods and Appendix 1). In order to facilitate the measurements, cells were treated with the division inhibitor cephalixin for 45 min to induce moderate elongation, yielding final cell length of $\sim 5 \mu\text{m}$ (Figure 1A). During an FCS measurement, the laser focus was positioned close to the polar region in the cell cytoplasm, and the fluorescence intensity in the confocal volume was measured over time (Figure 1 – figure supplement 2). For each individual cell, six subsequent acquisitions of 20 s each were performed at the same position. The autocorrelation function (ACF) of the fluorescence intensity fluctuations was independently calculated for each time interval and fitted to extract the mobility parameters of the fluorescent proteins. Although we initially considered both the Brownian diffusion and the anomalous diffusion models, the latter model proved to be considerably better in fitting the experimental data (Figure 1 – figure supplement 3). The anomalous diffusion model was therefore

used to determine the diffusion (or residence) time (τ_D) of a fluorescent molecule in the confocal volume and the anomalous diffusion exponent α for all ACFs (Figure 1B and Figure 1 – supplementary table 1 and figure supplement 2). The averaged values of τ_D and α for each individual cell were then calculated from these six individual acquisitions (Figure 1C and Figure 1 – figure supplement 4).

Despite their substantial intercellular variability, thus obtained mean values of the diffusion time were clearly different between protein constructs (Figure 1C and Figure 1 – supplementary table 1. We next plotted the mean values of $1/\tau_D$, which reflect protein mobility, against the molecular mass of protein constructs (Figure 1D). This dependence revealed a clear trend, where mobility of more than half of the constructs decreased uniformly with their molecular mass, while some exhibited much lower mobility than the other constructs of similar mass. In contrast, the anomalous diffusion exponent α showed no apparent dependence on the protein size, ranging from 0.8 to 0.86 for most of the constructs (Figure 1E). Notably, the few protein constructs with α of ~ 0.8 or lower were also among the ones with low mobility (Figure 1D and 1E, colored symbols).

Figure 1. Dependence of protein mobility in bacterial cytoplasm on molecular mass and cellular interactions.

(A) Examples of fluorescence microscopy images of *E. coli* cells expressing either sfGFP or the indicated sfGFP-tagged cytoplasmic proteins. Scale bar is 2 μ m. (B) Representative autocorrelation functions (ACFs) measured by FCS for the indicated protein constructs. Data were fitted using the anomalous diffusion model (solid lines). All ACF curves were normalized to their respective maximal values to facilitate comparison. (C) Diffusion times (τ_D) measured for the indicated protein constructs. Each dot in the box plot represents the value for one individual cell, averaged over six consecutive acquisitions (Figure 1 – figure supplement 2) *** $p < 0.0001$ in a two-tailed heteroscedastic t -test. Exact p -values for all significance analyses performed in this study could be found in Appendix 4. (D, E). Dependence of protein mobility ($1/\tau_D$) (D) and apparent anomaly of diffusion (α) (E) on molecular mass. Each symbol represents

the average value for all individual cells that have been measured for that particular construct and the error bars represent the standard error of the mean. Individual values are shown in Figure 1 – figure supplement 4 and the numbers of measured cells are shown in Appendix 5. Protein constructs with most aberrant mobility are highlighted in color and labeled. For fusions to ClpS, Map and DnaK, the values of $1/\tau_D$ and α for both original constructs (diamonds) and constructs where mutations were introduced to disrupt interactions (circles) are shown. For Map, two alternative amino acid substitutions that disrupt its interaction with the ribosome are shown (see Figure 1 – figure supplement 6). (F-H) Cartoons illustrating the cellular interactions that could affect mobility of ClpS (F), Map (G), and DnaK (H). ClpS engages with the ClpAP protease and with substrates, cartoon adapted from (Roman-Hernandez et al. 2011). Map interacts with the actively translating ribosomes, cartoon adapted from (Sandikci et al. 2013). DnaK interacts with unfolded client protein. Amino acidic residues that were mutated to disrupt interactions are highlighted (see text for details).

Figure 1 – source data 1. Original values used for plots in Figure 1C-E.

Macromolecular interactions reduce protein mobility

We reasoned that the main group of constructs that exhibit mobility close to the apparent mass-dependent upper limit represents proteins whose diffusion is only limited by macromolecular crowding, and that the aberrantly low $1/\tau_D$ and α of other constructs might be due to their specific interactions with other cellular proteins or protein complexes. Indeed, for three of these proteins (ClpS, Map, DnaK) such interactions are well characterized and can be specifically disrupted. ClpS is the adaptor protein that delivers degradation substrates to the protease ClpAP (Roman-Hernandez et al. 2011). The substrate-binding site of ClpS is constituted by three amino acid residues (D35, D36, H66) that interact with the N-terminal degron of target proteins (Figure 1F). If these residues are mutated into alanine, substrate binding *in vitro* is substantially reduced (Roman-Hernandez et al. 2011; Humbard et al. 2013). Additionally, ClpS directly docks to the hexameric ClpA. Consistently, we observed that while stability of the mutant construct

ClpS^{D35A_D36A_H66A}-sfGFP was not affected (Figure 1 – figure supplement 5), its mobility in a *ΔclpA* strain became significantly higher and less anomalous, with both $1/\tau_D$ and α reaching levels similar to those of other proteins of similar mass (Figure 1D and 1E and Figure 1 – figure supplement 6).

Similar results were obtained for the other two constructs. Map is the methionine aminopeptidase that cleaves the N-terminal methionine from nascent polypeptide chains (Solbiati et al. 1999). Map interacts with the negatively charged backbone of ribosomes through four positively charged lysine residues (K211, 218, 224, 226) located in a loop (Figure 1G). If these residues are mutated into alanine, the *in vitro* affinity of Map for the ribosomes is reduced (Sandikci et al. 2013). The mobility of Map-sfGFP was indeed much increased by alanine substitutions at all four lysine sites (Figure 1D and 1E and Figure 1 – figure supplement 5 and figure supplement 6). Interestingly, charge inversion of lysines to glutamic acid did not further increase Map-sfGFP mobility as was expected based on *in vitro* experiments (Sandikci et al. 2013).

DnaK is the major bacterial chaperone that binds to short hydrophobic polypeptide sequences, which become exposed during protein synthesis, membrane translocation or protein unfolding (Genevaux, Georgopoulos, and Kelley 2007). DnaK accommodates its substrate peptides inside a hydrophobic pocket (Figure 1H). The substitution of the valine residue 436 with bulkier phenylalanine creates steric hindrance that markedly decreases substrate binding to DnaK *in vitro* (Mayer, Rüdiger, and Bukau 2000), and both the $1/\tau_D$ and α of DnaK^{V436F}-sfGFP were significantly higher than for the correspondent wild-type construct (Figure 1D and 1E and Figure 1 – figure supplement 5 and figure supplement 6). Nevertheless, in this case the $1/\tau_D$ did not reach the levels of other proteins of similar molecular mass, which is likely explained by multiple interactions of

DnaK with other components of the cellular protein quality control machinery besides its binding to substrates (Kumar and Sourjik 2012).

Cytoplasmic diffusion rate is conserved between bacterial species

We then investigated whether sfGFP fusions to non-native proteins, originating from other bacteria, may show diffusion different from that of their *E. coli* counterparts. The existence of an organism-dependent “quinary” code of unspecific, short living interactions has been recently proposed in order to explain reduced mobility of heterologous human proteins in *E. coli* cytoplasm (Mu et al. 2017). Thus, we investigated the mobility of proteins from other Gram-negative proteobacteria *Yersinia enterocolitica*, *Vibrio cholerae*, *Caulobacter crescentus*, *Myxococcus xanthus* and from the Gram-positive bacterium *Bacillus subtilis* that are homologous to several freely-diffusing *E. coli* protein constructs (Figure 1 – figure supplement 7). Within this set of constructs, we observed no significant differences of $1/\tau_D$ values from their *E. coli* counterparts, except for AcnA from *M. xanthus* (Figure 1 – figure supplement 7A, C). Such lower mobility might be a sign of multimerization of *M. xanthus* AcnA, although cellular distribution of this construct was uniform. In contrast, most of these constructs showed slightly but significantly increased anomaly of diffusion (Figure 1 – figure supplement 7B, D), which might reflect increased “stickiness” of non-native proteins in *E. coli* cytoplasm.

Apparent anomaly of diffusion could be largely explained by confinement

When FCS measurements are performed in a confined space with dimensions comparable to those of the observation volume, such confinement may affect the apparent mobility of fluorescent

molecules (Gennerich and Schild 2000; Jiang et al. 2020). To investigate the effect of confinement on our FCS measurements, we performed Brownian dynamics simulations of FCS experiments with particles undergoing three-dimensional, purely Brownian diffusion inside a bacterial-cell like volume (Figure 2A *Inset*; see Materials and Methods). For the values of cell diameter commonly observed under our growth conditions, 0.8-0.9 μm , and over a wide range of particle diffusion coefficients, simulated autocorrelation functions could be indeed successfully fitted with the anomalous diffusion model, yielding an anomalous diffusion exponent of around 0.8-0.9 (Figure 2A and 2B). This made us hypothesize that the relatively small apparent deviation from Brownian diffusion in the fit, common to most constructs, may primarily reflect a confinement-induced effect rather than proper subdiffusion. In order to test this, we grew *E. coli* in presence of the inhibitor A22, that is known to affect the biosynthesis of bacterial cell wall and lead to increased cell width (Ouzounov et al. 2016). Indeed, the cell diameter (Figure 2C) and the anomalous diffusion exponent of sfGFP (Figure 2D) both significantly increased upon such A22 treatment. We conclude that the values of α above 0.8, as observed for most protein constructs, reflect Brownian diffusion with confinement rather than truly anomalous diffusion.

We thus derived an Ornstein-Uhlenbeck (OU) model for fitting FCS data, where the confinement of Brownian diffusing fluorescent particles within the width of the cell is approximated by trapping in a harmonic potential of the same width (Appendix 2). The two models fit the ACF of the Brownian dynamic simulations comparably well and better than the model of unconfined Brownian diffusion (Figure 2A and Figure 2 - figure supplement 1), with the OU model having one less free parameter than the anomalous diffusion model. The OU model directly estimates the ansatz diffusion coefficient with $\pm 5\%$ accuracy for the typical cell widths observed in our experiments (Figure 2 – figure supplement 2).

We then applied the OU model to re-fit the ACF data for all faster diffusing constructs and to estimate their Brownian diffusion coefficients. The OU proved accurate in fitting the experimental data, comparably to the anomalous diffusion model (Figure 2 – figure supplement 3). The dependence of D on molecular mass for this set of constructs was scaling as $(MM)^{-\beta}$ with $\beta = 0.56 \pm 0.05$ (Figure 2E), less steep compared to the previous estimates (Kumar, Mommer, and Sourjik 2010; Mika et al. 2010; Stracy et al. 2021) but still steeper than expected from the Stokes-Einstein relation, even when assuming $\beta = 0.4$ for not perfectly globular proteins (Figure 2E *Inset*) (Enright and Leitner 2005; Smilgies and Folta-Stogniew 2015). In order to elucidate whether part of this residual deviation may be accounted for by the specific shape of fusion constructs, where sfGFP is fused to the differently-sized target proteins by a short flexible linker, we further applied a previously derived model describing diffusion of such linked proteins (Appendix 3) (Agudo-Canalejo and Golestanian 2020). The dependence of D on molecular mass predicted by this linked-protein model seems indeed to better recapitulate our experimental data, particularly for smaller protein fusions (Figure 2E *Inset*). Nevertheless, this model still clearly overestimates D for larger protein fusions, suggesting that at least in this size range the dependence of D on molecular mass in bacterial cytoplasm deviates from the Stokes-Einstein relation.

Figure 2. Protein diffusion in bacterial cytoplasm corrected for confinement. (A) Representative ACFs of simulated fluorescence intensity fluctuations. Simulations were performed in a confined geometry of a cell with indicated length L and diameter d , and dimensions of the measurement volume w_0 and z_0 , representing an experimental FCS measurement (*Inset*; see Materials and Methods) for two different values of the ansatz diffusion coefficient. Solid lines are fits by the models of unconfined Brownian diffusion, anomalous diffusion and by the Ornstein-Uhlenbeck (OU) model of Brownian diffusion under confinement, as indicated. (B) The exponent α extracted from the fit of the anomalous diffusion model to the ACFs data that were simulated at different values of the cell diameter. Corresponding values of the diffusion coefficient are shown in Figure 2 – figure supplement 2. (C,D) *E. coli* cells

grown for ~ 4 hours either in absence or in presence of 1 $\mu\text{g/mL}$ of A22, show A22-dependent increase in the measured cell diameter (C) and higher values of the exponent α extracted from the fit to the ACF measurements (D). For consistency with other experiments, the cultures were also treated with cephalexin for 45 min. *** $p < 0.0001$; * $p < 0.05$ in a two-tailed heteroscedastic t -test. (E) Dependence of the diffusion coefficient calculated from fitting the experimental ACFs with the OU model of confined diffusion. Only the subset of apparently freely diffusing constructs from Figure 1D has been analyzed with the OU model (see also Figure 1 – supplementary table 1). Each circle represents the average value for all individual cells that have been measured for that particular construct, and the error bars represent the standard error of the mean. *Inset* shows the fit of the mass dependence with an inverse power law (solid blue line, exponent $\beta = 0.56 \pm 0.05$), and predictions of the Stokes-Einstein relation (black dashed line) and of the model describing diffusion of two linked globular proteins (solid yellow line), both with exponent $\beta = 0.4$.

Figure 2 – source data 1. Original values used for plots in Figure 2B-E.

Protein diffusion coefficients measured using FRAP or FCS are consistent

Since many previous measurements of protein diffusion in bacteria were performed using FRAP, we aimed to directly compare the results of FRAP and FCS measurements for a set of constructs of different mass. Importantly, we used the same growth conditions and microscopy sample preparation protocols as for the FCS experiments. The cells were photobleached in a region close to the pole, similar to the position that was used for the FCS experiment. The recovery of fluorescence was then followed for 11 seconds with the time resolution of 18 ms (Figure 3A). The diffusion coefficients were computed from the time course of recovery with the plugin for ImageJ, simFRAP (Blumenthal et al. 2015), which utilizes a simulation-based approach (Figure 3B). We observed very good correlation between both values of diffusion coefficients, although for most constructs the diffusion coefficients determined by FRAP were 5 to 30% lower than those obtained from the FCS data (Figure 3C and Figure 1 – supplementary table 1).

Figure 3. Comparison between protein diffusion coefficients measured by FCS and FRAP. (A) Examples of FRAP measurements for two different constructs, sfGFP and AcnA-sfGFP. A 3x3 pixels area close to one cell pole (red circle) was photobleached with a high intensity laser illumination for 48 ms and the recovery of fluorescence in the bleached area was monitored for 11 s with the time resolution of 18 ms. (B) Representative curves of fluorescence recovery in FRAP experiments and their fitting using simFRAP. The experimental data (colored dots) are used by the simFRAP algorithm to simulate the underlying diffusional process (colored lines). The simulation is then used to compute the diffusion coefficient. The simulation proceeds until the recovery curve reaches a plateau, therefore it is interrupted at a different time for each curve. (C) Correlation between the diffusion coefficients measured in FCS experiments (D_{FCS} , fitting with the OU model; data from Figure 2E) and in FRAP experiment (D_{FRAP} , fitting with simFRAP). Error bars represent the standard error of the mean.

Figure 3 – source data 1. Original values used for plot in Figure 3C.

348

349 **Effects of temperature, osmolarity and antibiotics on protein mobility**

350 Having established the assay of protein mobility, we selected several differently-sized constructs
 351 with high mobility to characterize the impact of environmental and cellular perturbations that can
 352 be experienced by bacterial cells. We first confirmed the previously well characterized (Konopka
 353 et al. 2006; Konopka et al. 2009; Mika et al. 2010; Liu et al. 2019) decrease in protein mobility in
 354 bacterial cytoplasm upon osmotic upshift. Exposing *E. coli* cells to increased ionic strength by
 355 addition of 100 mM NaCl indeed decreased mobility of sfGFP by 30% (Figure 4 – figure
 356 supplement 1), which is comparable to previously measured values (Konopka et al. 2009).

357 Next, we studied the effect of temperature on protein mobility in bacterial cytoplasm. According
 358 to the Stokes-Einstein equation, the diffusion of a particle directly depends on the system's
 359 temperature in Kelvin and on the viscosity of the fluid, which itself changes with temperature. In
 360 the biologically relevant range, the temperature sensitivity of diffusion is primarily determined by
 361 the temperature dependence of water viscosity. The measured increase in mobility of sfGFP and
 362 two other constructs, by approximately 20-25% between 25°C and 35°C (Figure 4A), agrees well
 363 with the expected temperature-dependent decrease in water viscosity over 10°C (Huber et al.
 364 2009). Importantly, growth temperature of *E. coli* culture had no apparent effect on protein
 365 mobility (Figure 4 – figure supplement 2), suggesting that – at least in the tested temperature range
 366 – *E. coli* lacks the growth-temperature dependent regulation of cytoplasmic viscosity that has been
 367 recently reported in the budding yeast (Persson, Ambati, and Brandman 2020).

368 Antibiotics that inhibit transcription (e.g., rifampicin) or translation (e.g., chloramphenicol) are
 369 well known to affect the spatial organization of bacterial chromosomes (Bakshi et al. 2014), but

their effects on the diffusive properties of the cytoplasm are less established. We observed that protein mobility significantly increased upon inhibition of RNA transcription by rifampicin treatment, roughly independently of protein size (Figure 4B and Figure 4 – figure supplement 3). In contrast, inhibition of protein translation by chloramphenicol slightly reduced mobility.

Figure 4. Effects of temperature and antibiotics on protein mobility. (A) The effect of environmental temperature on mobility of indicated protein constructs. As for other experiments, *E. coli* culture was grown at 37°C. (B) The effects of antibiotics treatment on mobility of indicated protein constructs. Chloramphenicol (Cam; 200 µg/mL), rifampicin (Rif; 200 µg/mL, in 0.1% v/v DMSO), or DMSO control (0.1% v/v) were added to growing *E. coli* culture 60 minutes prior to harvesting. *** p<0.0001; ** p<0.001; * p<0.05; NS: no statistically significant difference in a two-tailed heteroscedastic *t*-test.

Figure 4 – source data 1. Original values used for plots in Figure 4.

Effects of cell growth on protein mobility

In order to further investigate whether protein mobility might be affected by cell growth, we compared cells that were incubated at 35°C on agarose pads containing either only M9 salts or M9 salts plus glucose and casamino acids. The observed mobility was indeed significantly higher in

the presence of nutrients, and this increase was similar (~ 40%) for the two tested differently-sized constructs (Figure 5).

Figure 5. Effect of nutrient availability and growth on protein mobility. The mobility of indicated protein constructs was measured in cells incubated at 35°C on agarose pads containing either only M9 salts or M9 salts together with 20 mM glucose and 0.2% casamino acids (Glu + CA). Where indicated, chloramphenicol (Cam) was also added to the agarose pads. *** p<0.0001; ** p<0.001; * p<0.05 in a two-tailed heteroscedastic *t*-test.

Figure 5 – source data 1. Original values used for plots in Figure 5.

To distinguish the respective contributions of metabolic activity, and of biosynthesis and resulting cell growth, we incubated cells in presence of both nutrients and chloramphenicol on the agarose

pad. Similar to our previous experiments where chloramphenicol was added to the batch culture, its addition had no or little effect on protein mobility in absence of nutrients. In contrast, protein mobility in presence of nutrients was strongly affected by chloramphenicol treatment. Thus, the enhanced protein mobility in presence of nutrients largely depends on active protein production and cell growth. Nevertheless, even chloramphenicol-treated cells exhibited a moderate increase in protein mobility in presence of nutrients, indicating that the metabolic activity might also contribute to the overall effect.

Discussion

Bacteria rely on translational diffusion to deliver proteins and other macromolecules to their cellular destinations, including their reaction partners, and the diffusional properties of bacterial cytoplasm are therefore fundamental to the understanding of bacterial cell biology. Consequently, a number of studies have investigated protein mobility in bacteria, all showing strong effects of macromolecular crowding in the bacterial cytoplasm on diffusion (Konopka et al. 2006; Mullineaux et al. 2006; Konopka et al. 2009; Kumar, Mommer, and Sourjik 2010; Mika et al. 2010; Nenninger, Mastroianni, and Mullineaux 2010). Nevertheless, the relatively small number of proteins investigated in each of these previous studies, and the differences between strains, growth conditions and, particularly, between methodologies, limited general conclusions about protein mobility, even in the most-studied environment of *E. coli* cytoplasm. For example, combining data from different studies to determine the relation between size of a protein and its cytoplasmic diffusion coefficient yielded only uncertain estimates (Mika and Poolman 2011; Schavemaker, Boersma, and Poolman 2018). Such variability between different studies might be

further compounded by potentially profound effects on diffusion of size-independent protein properties such as surface charge (Schavemaker, Smigiel, and Poolman 2017). Similarly, it remains unclear whether a typical protein in the cytoplasm exhibits Brownian diffusion, as has been shown in few examples (Bakshi, Bratton, and Weisshaar 2011; English et al. 2011), or rather a subdiffusive behavior as common for proteins in eukaryotic cells (Di Rienzo et al. 2014; Sabri et al. 2020) and for bacterial mRNA-protein complexes (Lampo et al. 2017; Golding and Cox 2004), and what might be the possible causes of anomalous diffusion in bacteria.

Here, we addressed these questions by systematically investigating the diffusive behavior of a large set of fluorescent protein fusions to differently-sized cytoplasmic proteins of *E. coli*. We demonstrate that, despite their diverse cellular functions, the majority of studied proteins exhibit a rather uniform relation between their molecular mass and cytoplasmic mobility, with a clear upper bound on protein mobility at a given molecular mass. Furthermore, the apparent weak anomaly of diffusion observed in the FCS data analysis, with $\alpha \sim 0.85-0.9$ for most proteins, could be accounted for by confinement of the otherwise purely Brownian diffusing particles in the small volume of a bacterial cell. Similar conclusions have been drawn by previous SPT studies for several individual proteins (Bakshi, Bratton, and Weisshaar 2011; English et al. 2011). This interpretation was directly confirmed by performing FCS measurements in *E. coli* with an increased cell diameter, which indeed yielded $\alpha \sim 0.95$.

We therefore used a model of purely Brownian diffusion under confinement (Ornstein-Uhlenbeck model) to determine diffusion coefficients by directly fitting the autocorrelation functions of our FCS measurements. The obtained overall dependence of diffusion coefficients on the molecular mass of the fusion protein showed the exponent $\beta = 0.56$, steeper than predicted by the Stokes-Einstein relation, with $\beta = 0.33$ for fully compact proteins or $\beta = 0.4$ for the more realistic case

where proteins are assumed to be not entirely compact (Enright and Leitner 2005; Smilgies and Folta-Stogniew 2015). Nevertheless, at least for smaller constructs, the observed dependence of the diffusion coefficient on the molecular mass could be well reproduced when the specific shape of fusion constructs, where two roughly globular proteins are fused by a short linker, was taken into account along with their imperfect globularity (Agudo-Canalejo and Golestanian 2020). In contrast, the diffusion of larger proteins was still slower than calculated by this model.

Our analysis thus suggests that, despite the high crowdedness of the bacterial cytoplasm, the diffusion of typical cytoplasmic proteins in bacteria is mostly Brownian and can be well described by treating the cytoplasm as a viscous fluid, with only a moderate dependence of the effective viscosity on the size of diffusing proteins. Given the diffusion coefficient determined in our study for free sfGFP, $\sim 14 \mu\text{m}^2\text{s}^{-1}$, for small proteins this effective viscosity of bacterial cytoplasm is only approximately six times higher than in dilute solution (Potma et al. 2001). This diffusion coefficient for GFP is substantially larger than the values reported in the early studies that used FRAP (Elowitz Michael et al. 1999; Konopka et al. 2006; Mullineaux et al. 2006; Konopka et al. 2009; Kumar, Mommer, and Sourjik 2010; Mika et al. 2010; Nenninger, Mastroianni, and Mullineaux 2010), although it is consistent with other FCS and SPT studies (Meacci et al. 2006; English et al. 2011; Sanamrad et al. 2014; Diepold et al. 2017; Rocha et al. 2019). These differences are apparently due to the limitations of early FRAP analyses that generally underestimated protein mobility, rather than due to different spatial and temporal scales assessed by the two techniques, since our direct comparison between FCS and FRAP measurements yielded similar values of diffusion coefficients. Indeed, a more recent FRAP study also reported higher diffusion coefficients for GFP (Schavemaker, Smigiel, and Poolman 2017).

Several proteins in our set showed much lower mobility than expected from their size, and in some cases also clearly subdiffusive behavior. For three selected examples, such deviation could be explained by specific association of these proteins with other proteins or multiprotein complexes. This is consistent with theoretical studies suggesting that binding of diffusing molecules to crowders can lead to subdiffusion (Saxton 2007; Guigas and Weiss 2008). A similar explanation is likely to hold for the other aberrantly slow proteins.

Unspecific transient interactions might also explain the slightly subdiffusive behavior of sfGFP fusions to proteins from other bacteria in *E. coli* cytoplasm. However, this anomaly was weak and there was overall only little difference between the mobility of these non-native proteins and their similarly-sized *E. coli* homologues, in contrast to pronounced differences observed between bacterial and mammalian proteins (Mu et al. 2017). Thus, there is apparently little organism-specific adaptation of freely diffusing proteins to their “bacterial host”, with a possible exception of bacteria with very different pH or ionic strength of the cytoplasm (Schavemaker, Smigiel, and Poolman 2017), which might facilitate horizontal gene transfer among bacteria.

We further probed how the effective viscous properties of bacterial cytoplasm changed under different physicochemical perturbations, using a subset of proteins that showed fast diffusion for their molecular mass as “reporters” of effective viscosity. Consistent with the importance of macromolecular crowding and in agreement with previous results (Konopka et al. 2009), protein mobility decreased upon osmotic upshift that increases cytoplasmic crowding. In contrast, the effective cytoplasmic viscosity significantly (~20%) decreases upon treatment with rifampicin that inhibits transcription and thereby reduces the overall macromolecular crowding. This observation is consistent with recent SPT measurements (Wlodarski et al. 2017; Rotter et al. 2021), and it

agrees well with the relative contribution of RNA to the macromolecular composition of an *E. coli* cell (Cayley et al. 1991).

Despite multiple effects of environmental temperature on cellular processes, temperature dependence of the cytoplasmic viscosity in the tested range was similar to that of water and consistent with the Stokes-Einstein relation, decreasing by 20-30% for a temperature increase of 10°C (Huber et al. 2009). We observed no dependence of the effective cytoplasmic viscosity on growth temperature, in contrast to the homeostatic adaptation of bacterial membrane fluidity (Sinensky 1974) and of bacterial signaling (Oleksiuk et al. 2011; Almblad et al. 2021) to the growth temperature. Since growth-temperature dependent adaptation of the cytosolic viscosity was recently reported for budding yeast (Persson, Ambati, and Brandman 2020), it is surprising that such compensation apparently does not exist in *E. coli*. One possible explanation for this difference might be a broader range of growth temperatures for budding yeast *Saccharomyces cerevisiae* compared to *E. coli* and a stronger temperature effect on protein diffusion in the yeast cytosol. Of note, here we did not explore protein diffusion in thermally stressed *E. coli* cells, which might have more profound effects on the properties of bacterial cytoplasm as recently shown for *Listeria monocytogenes* (Tran et al. 2021).

Finally, we observed that protein mobility was significantly higher in actively growing cells. This “fluidizing” effect of growth seems to be primarily due to the biosynthetic processes, likely protein translation, or to cell growth itself, whereas the effect of metabolic activity in presence of nutrients was weaker although still significant. Thus, the observed phenomenon may be different from previously characterized energy-dependent fluidization of the bacterial cytoplasm that enables mobility of large multiprotein complexes but apparently does not affect free GFP (Montero Llopis

et al. 2012; Parry et al. 2014). The interplay between these energy-, metabolism- and growth-dependent effects on diffusional properties of bacterial cytoplasm remains to be investigated. Summarizing, here we present a comprehensive study of the diffusional properties of proteins in the cytoplasm of *E. coli*, combining FCS measurements with theoretical modeling of diffusion in a confined geometry of a bacterial cell. This analysis enabled us to distinguish a large set of proteins with essentially Brownian diffusion, which could be used to characterize the effective cytoplasmic viscosity over a range of molecular masses and its dependence on macromolecular crowding, protein interactions, environmental temperature and cell growth.

Materials and methods

Bacterial strains, plasmids and media

All strains and plasmids used in this study are listed in Figure 1 – supplementary table 2. All experiments were performed in the *Escherichia coli* strain W3110 (Serra et al. 2013). Genes of interest were amplified by PCR using Q5 polymerase (New England Biosciences) and cloned along with sfGFP using Gibson assembly (Gibson et al. 2009) into pTrc99A vector (Amann, Ochs, and Abel 1988), under control of the *trc* promoter inducible by isopropyl β -D-1-thiogalactopyranoside (IPTG). In all cases sfGFP was fused at the C-terminus of the protein of interest with a GGGGS linker. The stability of the fusion constructs was verified by gel electrophoresis and immunoblotting using an anti-GFP primary antibody (JL-8 monoclonal, Takara). Point mutations were introduced by site-directed mutagenesis (New England Biosciences). *ΔclpA* was generated by transferring the kanamycin resistance cassette from the corresponding mutant in the Keio collection (Baba et al. 2006) by P1 transduction. The cassette

was further removed by FLP recombinase carried on the temperature-sensitive plasmid pCP20 (Cherepanov and Wackernagel 1995).

E. coli cultures were grown in M9 minimal medium (48 mM Na₂HPO₄, 22 mM KH₂PO₄, 8.4 mM NaCl, 18.6 mM NH₄Cl) supplemented with 0.2% casamino acids, 0.4% glucose and 100 µg/mL ampicillin for selection. The overnight cultures were diluted to OD₆₀₀ = 0.035 and grown for 3.5 hours at 37°C and 200 rpm shaking. Cultures were treated for additional 45 minutes, under the same temperature and shaking conditions, with 10 µg/mL cephalixin and with 0 - 15 µM IPTG (Figure 1 – supplementary table 1), to induce expression of the fluorescent protein constructs. Where indicated, cultures were further incubated with 200 µg/mL rifampicin, 200 µg/mL chloramphenicol or DMSO as a mock treatment for 1 hour under the same temperature and shaking conditions.

FCS data acquisition

Cells were harvested by centrifugation at 7000g for 3 minutes and washed 3 times in tethering buffer (10 mM K₂HPO₄, 10 mM KH₂PO₄, 1 µM methionine, 10 mM sodium lactate, buffered with NaOH to pH 7). 2.5 µL of bacterial cells were then spread on a small 1% agarose pad prepared in tethering buffer salts (10 mM K₂HPO₄, 10 mM KH₂PO₄ buffered with NaOH to pH 7), unless differently stated. Imaging was performed on Ibidi 2-well µ-Slides (#1.5H, 170 µm ± 5 µm). After the 45 minutes treatment with cephalixin, length of most bacterial cells was in a range of 4-8 µm. For consistency, only cells of 5 ± 1 µm in length were selected for FCS experiments.

FCS measurements were performed on a LSM 880 confocal laser scanning microscope (Carl Zeiss Microscopy) using a C-Apochromat 40×/1.2 water immersion objective selected for FCS. sfGFP

was excited with a 488 nm Argon laser (25 mW) and fluorescence emission was collected from 490 to 580 nm. Each sample was equilibrated for at least 20 minutes at 25°C (or 35°C when specified), on the stage of the microscope and measurements were taken at the same temperature. FCS measurements were acquired within 60 minutes from the sample preparation. The pinhole was aligned on a daily basis, by maximizing the fluorescence intensity count rate of an Alexa488 (Invitrogen) solution (35 nM) in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4). The coverslip collar adjustment ring of the water immersion objective was also adjusted daily, maximizing the fluorescence intensity signal and the brightness of Alexa 488. The laser power was adjusted in order to obtain molecular brightness (i.e. photon counts per second per molecule, cpsm) of 10 kcpsm for Alexa 488, using the ZEN software (Carl Zeiss Microscopy). The brightness of Alexa 488 was used as a daily reference to ensure constant laser power and adjusting it using the software-provided laser power percentage whenever necessary (range over the entire set of measurements was 0.11 – 0.18%). Before each measurement session, we acquired three sequential FCS measurements of Alexa488 in PBS, to verify the reproducibility of the confocal volume shape and size. The ratio between axial and lateral beam waist $S = \frac{z_0}{\omega_0} = 8.0 \pm 0.2$ was obtained from a Brownian fit of the Alexa 488 autocorrelation curves using the ZEN software. For the lateral beam waist, we obtained $\omega_0 = 0.186 \pm 0.001 \mu\text{m}$, calculated from the diffusion time $\tau_D = 20.9 \pm 0.11 \mu\text{s}$ obtained from the Brownian fit, being

$$D = \frac{\omega_0^2}{4\tau_D} \quad (1)$$

and being $D_{Alexa488} = 414 \mu\text{m}^2/\text{s}$ at 25°C (Petrov et al. 2006).

For the FCS measurements *in vivo*, the laser was positioned at the center of the short length axis and typically 0.8-1 μm from one of the cell poles along the long axis. The positioning was chosen

to maintain distance from the cell membranes and to keep the confocal volume possibly away from the bacterial chromosome. For each cell, six sequential fluorescence intensity acquisitions of 20 seconds each were performed on the same spot (Figure 1 – figure supplement 2). The laser power used for measurements *in vivo* was fixed to a value about 7 times lower than for Alexa488 in PBS, in order to reduce photobleaching. Confocal images of the selected cell were routinely acquired before and after the FCS measurement to verify focal (*z*) and positioning (*xy*) stability (see Appendix 1 for additional information on the FCS measurements).

FCS data analysis

Due to the small size of bacterial cells, fluorescence intensity traces are affected by photobleaching (Appendix 1). The effect of photobleaching on autocorrelation curves was corrected by detrending the long-time fluorescence decrease of each of the six fluorescence intensity traces using an ImageJ plugin (Jay Unruh, <https://research.stowers.org/imagejplugins/index.html>, Stowers Institute for Medical Research, USA). The plugin calculates the autocorrelation function (ACF) from each fluorescence intensity trace, correcting it for the photobleaching effect by approximating the decreasing fluorescence intensity trend with a multi-segment line (the number of segments was fixed to two). We obtained almost identical ACFs correcting for photobleaching effects by local averaging (Appendix 1 - figure 3) using the FCS-dedicated software package Fluctuation Analyzer (Wachsmuth et al. 2015). In both cases, ACFs were calculated starting at 2 μ s, since at times shorter than 2 μ sec, ACFs can be significantly affected by the GaAsp photomultipliers afterpulsing.

For each FCS measurement, we fitted all the six ACFs, calculated using the multi-segment detrending method, with a three-dimensional anomalous diffusion model that includes one diffusive component and one blinking component due to the protonation-deprotonation of the chromophore of sfGFP, according to the Equation (2):

$$G(\tau) = G_{\infty} + \frac{1}{N} \left(\frac{1 - F_P + F_P e^{-\frac{\tau}{\tau_P}}}{1 - F_P} \right) \frac{1}{1 + \left(\frac{\tau}{\tau_D} \right)^{\alpha} \sqrt{1 + \frac{1}{S^2} \left(\frac{\tau}{\tau_D} \right)^{\alpha}}} \quad (2)$$

where N is the average number of particles in the confocal volume, F_P is the fraction of particles in the non-fluorescent state, τ_P is the protonation-deprotonation lifetime at pH 7.5, $S = \frac{z_0}{\omega_0}$, the aspect ratio of the confocal volume with z_0 and ω_0 being the axial and lateral beam waists, τ_D is the diffusion time in the confocal volume, α is the anomalous diffusion exponent, and G_{∞} is the offset of the autocorrelation function. The protonation-deprotonation lifetime (τ_P) for sfGFP was fixed to 25 μ s according to FCS measurements for sfGFP in PBS at pH 7.5 (Cotlet et al. 2006). The aspect ratio of the confocal volume was fixed to $S = 8$ in the fittings to be consistent with the experimental calibration (see above). All other parameters were left free. For each FCS measurement, we calculated the average diffusion time τ_D and the average anomalous diffusion exponent α based on the autocorrelation curves of the six sequential fluorescence intensity traces. Importantly, no significant trend in τ_D or α was apparent when comparing the six sequential autocorrelation functions acquired for a given bacterial cell (Appendix 1 - figure 4). Fitting to the anomalous diffusion model was performed using the Levenberg-Marquardt algorithm in the FCS analysis-dedicated software QuickFit 3.0 developed by Jan Wolfgang Krieger and Jörg Langowski (Deutsches Krebsforschungszentrum, Heidelberg, <https://github.com/jkriege2/QuickFit3>). Identical results were obtained when fitting the data with OriginPro.

Alternatively, the autocorrelation functions were fitted by the Ornstein-Uhlenbeck model (Appendix 2) according to Equation (3):

$$G(\tau) = G_{\infty} + \frac{1}{N} \left(\frac{1-F_P+F_P e^{-\frac{\tau}{\tau_P}}}{1-F_P} \right) \left(1 + 2 \frac{\sigma^2}{\omega_0^2} \frac{1-e^{-\frac{1}{2} \frac{\omega_0^2}{\sigma^2} \tau}}{1+\frac{1}{8} \frac{\omega_0^2}{\sigma^2}} \right)^{-\frac{1}{2}} \left(1 + \frac{\tau}{\tau_D} \right)^{-\frac{1}{2}} \left(1 + 2 \frac{\sigma^2}{S^2 \omega_0^2} \frac{1-e^{-\frac{1}{2} \frac{\omega_0^2}{\sigma^2} \tau}}{1+\frac{1}{8} \frac{S^2 \omega_0^2}{\sigma^2}} \right)^{-\frac{1}{2}} \quad (3)$$

where S and τ_P were fixed to the same values mentioned for equation (2), ω_0 was fixed to 0.19 and σ was fixed to $d/2=0.42 \mu\text{m}$, being d the typical diameter of an *E. coli* cell (see Ornstein-Uhlenbeck model validation paragraph). Fitting to the Ornstein-Uhlenbeck model was performed with OriginPro.

FRAP data acquisition and analysis

Cells for FRAP experiments were grown and prepared for imaging following the same protocol as for the FCS measurements. Due to the higher sensitivity of FCS at low fluorophore concentrations, several fusion constructs required higher induction by IPTG (Figure 1 – supplementary table 1) to obtain fluorescence intensity suitable for FRAP. The same LSM 880 confocal microscope, including objective and light path was used for FRAP as for the FCS measurements. The bacterial cell was imaged at 40×40 pixels with $30 \times$ zoom (pixel size $0.177 \mu\text{m}$) with a pixel dwell time of $3.15 \mu\text{s}$. First, 15 pre-bleaching frames were acquired at 2% laser power, subsequently the photobleaching was performed on a 3×3 pixels area on one cell pole with 100% laser power for a total of 48 ms and 584 post-bleaching frames were acquired to monitor the fluorescence recovery. We observed that the mobile fraction for all constructs was >0.9 . FRAP measurements were analyzed using simFRAP (Blumenthal et al. 2015), an ImageJ plugin based on a simulation

approach implemented in a fast algorithm, which bypasses the need of using analytical models to interpolate the data. The simFRAP algorithm simulates two-dimensional random walks in each pixel, using the first image acquired after bleaching to define initial and boundary conditions, and it resolves numerically the diffusion equation by iterative simulation. The frame time and pixel size were fixed respectively to 0.018 s and 0.177 μm , and the target cell and the bleached region were defined as ImageJ ROIs (regions of interest). Of note, we used the target cell itself as a reference to compensate for the gradual bleaching during the measurement, as done previously (Kumar, Mommer, and Sourjik 2010). This enabled us to achieve the highest possible temporal resolution, by reducing the acquisition area to a single *E. coli* cell. The FRAP derived diffusion coefficient D_{FRAP} was directly obtained as output of the plugin.

Brownian dynamics simulations

We performed Brownian dynamics simulations of uncorrelated point particles under confinement. The $N = 50$ fluorescent particles performed a random walk with steps taken from a Gaussian distribution of width $\sqrt{2D\Delta t}$, with D the free diffusion coefficient and $\Delta t = 10^{-6}$ s the simulation step. Confinement was imposed by redrawing the random steps that moved out of the confinement volume. Imposing elastic reflections on the walls yielded identical results. The confinement volume was assumed to be a cylinder of diameter d and length $(L-d)$ closed at both ends by hemispheric caps of diameter d , idealizing the shape of *E. coli*. The cell length was fixed to $L = 5$ μm . The diameter was varied in the range $d = [0.7, 1]$ μm . The collected fluorescence intensity was computed at each time step assuming a Gaussian intensity profile of the laser beam, $I(t) = \sum_{i=1}^N I_G(\mathbf{r}_i(t) - \mathbf{r}_0)$ with $\mathbf{r}_i(t)$ the position of particle i , $\mathbf{r}_0$ the center of the confocal volume and

661 $I_G(\mathbf{r} = (x, y, z)) = \exp\left(-2\left(\frac{x^2 + y^2}{\omega_0^2} + \frac{z^2}{z_0^2}\right)\right)$, with $\omega_0 = 200$ nm and $z_0 = 800$ nm the lateral and
662 axial widths of the confocal volume. The normalized intensity autocorrelation $C(dt) =$
663 $\frac{\langle I(t+dt)I(t) \rangle}{\langle I(t+dt) \rangle \langle I(t) \rangle} - 1$ is computed for logarithmically spaced lag times dt , to reflect experimental
664 practices. The center of the confocal volume was chosen in the center of the cell along the y and
665 z axes and 1 μm away from the edge of the cell along the longitudinal x axis of the cell, similarly
666 to experimental conditions. The intensity autocorrelation function was finally multiplied by an
667 exponential decay, $\left(1 + 0.1 * \exp\left(-\frac{dt}{\tau_H}\right)\right)/1.1$ with $\tau_H = 25$ μs , to mimic the blinking component
668 due to the protonation-deprotonation process of sfGFP, before fitting with the different models of
669 diffusion. The code used for this simulation is available in GitHub
670 (https://github.com/croelmiyn/Simulation_FCS_in_Bacteria) and via
671 DOI: 10.5281/zenodo.5940484.

672

673 **Validation of fitting by the Ornstein-Uhlenbeck model**

674 We first estimated the relation between the width σ of the potential well and the diameter d of the
675 bacteria by fitting the ACF of the Brownian simulations with the OU model, fixing all parameters
676 except σ to their ansatz values. The best fit was obtained for $\sigma \simeq d/2$ over the whole range of
677 tested parameters. To mimic the fit procedure of experimental data and evaluate the accuracy of
678 the diffusion coefficient estimation by the OU model (Fig. 2C), we then fixed $\sigma = d/2$, and ω_0
679 and z_0 to their ansatz values, since they are measured independently in experiments, whereas the
680 diffusion coefficient, number of particles N in the confocal volume, fraction of triplet excitation
681 and background noise were taken as free parameters.

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Competing Interests

The authors declare no competing interests.

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959 **Supplementary files**

960 This article contains the following supplementary files:

961 Figure 1 – supplementary table 1

962 Figure 1 – supplementary table 2

963 Figure 1 – figure supplement 1

964 Figure 1 – figure supplement 2

965 Figure 1 – figure supplement 3

966 Figure 1 – figure supplement 4

967 Figure 1 – figure supplement 5

968 Figure 1 – figure supplement 6

969 Figure 1 – figure supplement 7

970 Figure 2 – figure supplement 1

971 Figure 2 – figure supplement 2

972 Figure 2 – figure supplement 3

973 Figure 4 – figure supplement 1

974 Figure 4 – figure supplement 2

975 Figure 4 – figure supplement 3

- 976 Appendix 1: Notes on the acquisition and analysis protocols for FCS measurements in bacterial
- 977 cells
- 978 Appendix 2: Ornstein-Uhlenbeck model for confinement effect in FCS measurements
- 979 Appendix 3: Effective diffusion coefficient of two linked proteins
- 980 Appendix 4: Exact p-values for each figure
- 981 Appendix 5: Numerosity of the constructs and conditions for each experiment
- 982 Source data: Source data files and raw immunoblot images.

Supplementary figures

Figure 1 – supplementary table 1. sfGFP-tagged library of cytoplasmic proteins

Protein name	Molecular mass of sfGFP-construct	Biological function	IPFG concentration for FCS (for FRAP)	Number of cells (FCS)	t_0 (μ s; avg. $\pm$ SE)	α (Avg. $\pm$ SE)	Diffusion coefficient, FCS (μ m ² /s; Avg. $\pm$ SE)	Number of cells (FRAP)	Diffusion coefficient, FRAP (μ m ² /s; Avg. $\pm$ SE)
<i>sgGFP</i>	26.9	-	5 μ M (15 μ M)	42	580 $\pm$ 15	0.86 $\pm$ 0.01	14.4 $\pm$ 0.4	11	11.3 $\pm$ 1.3
<i>YggX</i>	39.2	Probable Fe (2+)-utilizing protein	5 μ M (5 μ M)	8	611 $\pm$ 19	0.85 $\pm$ 0.01	12.9 $\pm$ 0.4	10	9.4 $\pm$ 1.6
<i>CtpS</i>	39.2	ATP-dependent Ctp protease adapter protein	0 μ M	11	1054 $\pm$ 33	0.75 $\pm$ 0.01			
<i>FolK</i>	45.1	2-aminop-4-hydroxy-6-hydroxymethylidihydroxy-4-thiophosphokinase	0 μ M	8	734 $\pm$ 24	0.87 $\pm$ 0.01	11.6 $\pm$ 0.4		
<i>Ctr</i>	45.2	Component of glucose-specific phosphotransferase enzyme IIA	0 μ M	14	1065 $\pm$ 36	0.87 $\pm$ 0.01			
<i>UbcC</i>	45.7	Chorismate pyruvate-lyase	15 μ M	14	1140 $\pm$ 58	0.87 $\pm$ 0.01			
<i>ThpR</i>	46.9	RNA 2,3'-cyclic phosphodiesterase			Discarded due to instability of sfGFP-construct				
<i>CoaE</i>	49.6	Dehydrophospho-CoA kinase	0 μ M	11	854 $\pm$ 47	0.87 $\pm$ 0.01	9.8 $\pm$ 0.6		
<i>Adk</i>	50.6	Adenylate kinase	5 μ M (15 μ M)	23	802 $\pm$ 26	0.88 $\pm$ 0.00	10.6 $\pm$ 0.4	16	9.8 $\pm$ 1.5
<i>Cmk</i>	51.7	Cytidylate kinase	5 μ M	16	1163 $\pm$ 58	0.87 $\pm$ 0.01			
<i>NegD</i>	54.1	Ribonucleotide monophosphatase			Discarded due to non-uniform protein localization				
<i>KdsB</i>	54.6	3-deoxy-nmno-oculosonate cytidyltransferase	0 μ M	11	1659 $\pm$ 70	0.84 $\pm$ 0.01			
<i>Map</i>	56.3	Methionine aminopeptidase	0 μ M	20	1830 $\pm$ 78	0.81 $\pm$ 0.01			
<i>MmuM</i>	60.4	Homocysteine S-methyltransferase	5 μ M	14	2241 $\pm$ 138	0.73 $\pm$ 0.01			
<i>RibA</i>	60.8	Pyrimidine-specific ribonucleoside hydrolase			Discarded due to non-uniform protein localization				
<i>PurE</i>	60.8	2-dehydropanthoate 2-reductase	0 μ M (5 μ M)	18	1059 $\pm$ 26	0.85 $\pm$ 0.01	7.8 $\pm$ 0.2	11	5.2 $\pm$ 0.6
<i>SolA</i>	67.9	N-methyl-L-tryptophan oxidase	0 μ M	7	795 $\pm$ 31	0.82 $\pm$ 0.01	9.9 $\pm$ 0.5		
<i>Pgl</i>	68.1	Phosphoglycerate kinase	0 μ M	16	991 $\pm$ 41	0.90 $\pm$ 0.01	8.6 $\pm$ 0.3		
<i>EmcC</i>	69.9	Isochorismate synthase	15 μ M	15	1777 $\pm$ 119	0.82 $\pm$ 0.01			
<i>AroA</i>	73.1	3-phosphoshikimate 1-carboxyvinyltransferase	0 μ M	9	995 $\pm$ 69	0.86 $\pm$ 0.01	8.7 $\pm$ 0.7		
<i>ThiC</i>	74.1	Threonine synthase	0 μ M	14	908 $\pm$ 28	0.87 $\pm$ 0.01	9.1 $\pm$ 0.3		
<i>MurF</i>	74.4	UDP-N-acetylglucosamine-6-phosphate-4-epimerase	0 μ M	7	1008 $\pm$ 76	0.85 $\pm$ 0.02	8.3 $\pm$ 0.7		
<i>DsdA</i>	74.9	D-serine dehydratase	0 μ M	14	1017 $\pm$ 53	0.89 $\pm$ 0.01	8.4 $\pm$ 0.4	10	7.8 $\pm$ 0.7
<i>HemN</i>	79.7	Oxygen-independent coproporphyrinogen III oxidase	0 μ M	13	1262 $\pm$ 54	0.86 $\pm$ 0.01	6.7 $\pm$ 0.4		
<i>PcpD</i>	80.9	2-methylcitrate dehydratase	0 μ M	12	1866 $\pm$ 140	0.84 $\pm$ 0.01			
<i>DnaK</i>	96.0	Molecular chaperone	5 μ M	10	2296 $\pm$ 78	0.76 $\pm$ 0.01			
<i>MalZ</i>	96.0	Maltolectin glucosylase	0 μ M	9	3725 $\pm$ 229	0.77 $\pm$ 0.01			
<i>GleB</i>	107.5	Malate synthase G	5 μ M (15 μ M)	16	1315 $\pm$ 45	0.86 $\pm$ 0.01	6.4 $\pm$ 0.2	10	6.7 $\pm$ 1.1
<i>MeE</i>	111.7	5-methyltetrahydropteroyl-5-methyltetrahydrohomocysteine methyltransferase	0 μ M	8	1137 $\pm$ 53	0.87 $\pm$ 0.01	7.4 $\pm$ 0.3		
<i>Lea5</i>	124.2	Leucine-tRNA ligase	0 μ M	14	1637 $\pm$ 75	0.86 $\pm$ 0.01	5.1 $\pm$ 0.2		
<i>AcsA</i>	124.7	Acetate hydratase A	5 μ M (15 μ M)	19	1415 $\pm$ 56	0.86 $\pm$ 0.01	6.1 $\pm$ 0.2	10	4.3 $\pm$ 0.4
<i>MetH</i>	163.0	Methionine synthase	0 μ M (5 μ M)	9	1402 $\pm$ 45	0.81 $\pm$ 0.01	5.8 $\pm$ 0.1	15	4.0 $\pm$ 0.5

986 **Figure 1 – supplementary table 2. Strains and plasmids used in this study**

Strain or plasmid	Relevant genotype or phenotype	Reference or source
Strains		
<i>E. coli</i> W3110	W3110 derivative with functional RpoS [rpoS396(Am)]	(Serra Diego et al.)
NB63	W3110 Δ clpA	This work
Plasmids		
pTrec99A	Amp ^r ; expression vector; pBR ori; <i>trc</i> promoter, IPTG inducible	(Amann, Ochs, and Abel 1988)
pCP20	Amp ^r , Cam ^r , <i>flp</i>	(Cherepanov and Wackernagel 1995)
pNB1	Amp ^r ; <i>sfGFP</i> in pTrec99A	This work
pNB3	Amp ^r ; <i>Adk-sfGFP</i> in pTrec99A	This work
pNB4	Amp ^r ; <i>CoaE-sfGFP</i> in pTrec99A	This work
pNB5	Amp ^r ; <i>Cmk-sfGFP</i> in pTrec99A	This work
pNB6	Amp ^r ; <i>Pgk-sfGFP</i> in pTrec99A	This work
pNB7	Amp ^r ; <i>MmuM-sfGFP</i> in pTrec99A	This work
pNB8	Amp ^r ; <i>PrpD-sfGFP</i> in pTrec99A	This work
pNB9	Amp ^r ; <i>DsdA-sfGFP</i> in pTrec99A	This work
pNB11	Amp ^r ; <i>GlcB-sfGFP</i> in pTrec99A	This work
pNB13	Amp ^r ; <i>HemN-sfGFP</i> in pTrec99A	This work
pNB14	Amp ^r ; <i>Map^{WT}-sfGFP</i> in pTrec99A	This work
pNB15	Amp ^r ; <i>ThrC-sfGFP</i> in pTrec99A	This work
pNB16	Amp ^r ; <i>MalZ-sfGFP</i> in pTrec99A	This work
pNB17	Amp ^r ; <i>EntC-sfGFP</i> in pTrec99A	This work
pNB18	Amp ^r ; <i>ThpR-sfGFP</i> in pTrec99A	This work
pNB19	Amp ^r ; <i>AroA-sfGFP</i> in pTrec99A	This work
pNB20	Amp ^r ; <i>ClpS^{WT}-sfGFP</i> in pTrec99A	This work
pNB21	Amp ^r ; <i>Crr-sfGFP</i> in pTrec99A	This work
pNB22	Amp ^r ; <i>KdsB-sfGFP</i> in pTrec99A	This work
pNB23	Amp ^r ; <i>LeuS-sfGFP</i> in pTrec99A	This work
pNB24	Amp ^r ; <i>MurF-sfGFP</i> in pTrec99A	This work
pNB25	Amp ^r ; <i>NagD-sfGFP</i> in pTrec99A	This work
pNB26	Amp ^r ; <i>RihA-sfGFP</i> in pTrec99A	This work
pNB27	Amp ^r ; <i>SolA-sfGFP</i> in pTrec99A	This work
pNB28	Amp ^r ; <i>UbiC-sfGFP</i> in pTrec99A	This work
pNB29	Amp ^r ; <i>PanE-sfGFP</i> in pTrec99A	This work
pNB30	Amp ^r ; <i>FolK-sfGFP</i> in pTrec99A	This work
pNB39	Amp ^r ; <i>AcnA-sfGFP</i> in pTrec99A	This work
pNB40	Amp ^r ; <i>MetE-sfGFP</i> in pTrec99A	This work
pNB42	Amp ^r ; <i>MethH-sfGFP</i> in pTrec99A	This work
pNB44	Amp ^r ; <i>YggX-sfGFP</i> in pTrec99A	This work
pNB45	Amp ^r ; <i>Adk^{Cc}-sfGFP</i> in pTrec99A	This work
pNB46	Amp ^r ; <i>Adk^{Le}-sfGFP</i> in pTrec99A	This work
pNB47	Amp ^r ; <i>Adk^{Me}-sfGFP</i> in pTrec99A	This work
pNB48	Amp ^r ; <i>AcnA^{Ms}-sfGFP</i> in pTrec99A	This work
pNB49	Amp ^r ; <i>AcnA^{Le}-sfGFP</i> in pTrec99A	This work
pNB51	Amp ^r ; <i>Adk^{Es}-sfGFP</i> in pTrec99A	This work
pNB52	Amp ^r ; <i>AcnA^{Bs}-sfGFP</i> in pTrec99A	This work
pNB54	Amp ^r ; <i>Adk^{Ve}-sfGFP</i> in pTrec99A	This work
pNB56	Amp ^r ; <i>Pgk^{Cc}-sfGFP</i> in pTrec99A	This work
pNB58	Amp ^r ; <i>Pgk^{Ve}-sfGFP</i> in pTrec99A	This work
pNB59	Amp ^r ; <i>Pgk^{Ms}-sfGFP</i> in pTrec99A	This work
pNB60	Amp ^r ; <i>AcnA^{Le}-sfGFP</i> in pTrec99A	This work
pNB61	Amp ^r ; <i>DnaK^{WT}-sfGFP</i> in pTrec99A	This work
pNB62	Amp ^r ; <i>Map^{K211E_K218E_K224E_K226E}-sfGFP</i> in pTrec99A	This work
pNB63	Amp ^r ; <i>Map^{K211A_K218A_K224A_K226A}-sfGFP</i> in pTrec99A	This work
pNB64	Amp ^r ; <i>DnaK^{V36F}-sfGFP</i> in pTrec99A	This work
pNB66	Amp ^r ; <i>ClpS^{D35A_D36A_H66A}-sfGFP</i> in pTrec99A	This work

987

Figure 1 – figure supplement 1. Expression analysis for all *E. coli* protein constructs made in this study.

Expression of proteins was analyzed by SDS-PAGE and immunoblotting using a primary antibody specific for GFP.

All fusion proteins displayed a dominant band corresponding to the expected molecular mass of the full-length fusion

(Figure 1 – supplementary table 1).

Figure 1 – figure supplement 1 – source data 1-10. Raw images of immunoblots used for Figure 1 – figure

supplement 1.

Figure 1 – figure supplement 2. Workflow of a typical FCS experiment. The focus of the confocal microscope is positioned near one pole in the cell of interest, and six subsequent acquisitions of the fluorescence intensity of 20 seconds each are performed. The example for sfGFP shows only traces for R1, R3 and R5 measurements (different colors). The autocorrelation functions (ACFs) are then calculated independently for each acquisition. The values for τ_D and α are extracted independently from the fit to each ACF using the anomalous diffusion model (solid lines) and then averaged to obtain the τ_D and α for each individual cell.

Figure 1 – figure supplement 3. Comparison between fits of the experimental data with Brownian and anomalous diffusion models. The experimental data (here the example for the R3 measurement from Figure 1 – figure supplement 2) were fitted by the model of free Brownian diffusion and by the anomalous diffusion model as indicated by different colors (upper panel), with the corresponding values of residuals (lower panel).

Figure 1 – figure supplement 4 – source data 1. Original values used for plots in Figure 1 – figure supplement 4.

Figure 1 – figure supplement 5. Expression analysis for the mutants with impaired interactions. The expression of indicated point mutants of ClpS, DnaK and Map was analyzed by SDS-PAGE and immunoblotting using a primary antibody specific for GFP. All mutants displayed a dominant band corresponding to the expected molecular mass of the full-length fusion, and comparable to that of the wild-type counterpart. ClpS^{D35A_D36A_H66A}-sfGFP was measured in the same $\Delta clpA$ background as subsequently used for the FCS experiments.

Figure 1 – figure supplement 5 – source data 1-2. Raw images of immunoblots used for Figure 1 – figure supplement 5.

1020

1021 **Figure 1 – figure supplement 6. Mobility of ClpS, Map and DnaK and of indicated mutants with disrupted**
 1022 **protein interactions.** Each dot in the box plot represents the values of τ_D (A) and α (B) for one individual cell. ClpS
 1023 mutant was measured in $\Delta clpA$ background. *** $p < 0.0001$; * $p < 0.05$; NS: no statistically significant difference in a
 1024 two-tailed heteroscedastic t -test.

1025 **Figure 1 – figure supplement 6 – source data 1.** Original values used for plots in Figure 1 – figure supplement 6.

Figure 1 – figure supplement 7. Mobility of homologous proteins from other bacterial species in *E. coli*. The mobility (A) and anomalous diffusion exponent (B) of sfGFP fusions to homologues of Adk, Pgk and AcnA from indicated bacterial species (*E.c.* = *Escherichia coli*; *Y.e.* = *Yersinia enterocolitica*; *V.c.* = *Vibrio cholerae*; *C.c.* = *Caulobacter crescentus*; *M.x.* = *Myxococcus xanthus*; *B.s.* = *Bacillus subtilis*) compared with that of their counterpart from *E. coli*. *** $p < 0.0001$; ** $p < 0.001$; * $p < 0.05$ in a two-tailed heteroscedastic *t*-test. When not indicated, no statistically significant difference is observed. (C, D) Mass dependence of diffusion parameters of these sfGFP-constructs. Each symbol represents the average value for all individual cells that have been measured for that construct and the error bars represent the standard error of the mean.

Figure 1 – figure supplement 7 – source data 1. Original values used for plots in Figure 1 – figure supplement 7.

Figure 2 – figure supplement 1. Residuals of fitting the simulated ACFs with different models. Simulated fluorescence intensity ACF data were fitted by the models of anomalous and Brownian diffusion, as well as by the OU model of Brownian diffusion under confinement, as indicated.

Figure 2 – figure supplement 2. Diffusion coefficients fitted from simulation data. Diffusion coefficients computed ($D = \omega_0^2/4\tau_D$) from the diffusion times extracted from the fit of the Brownian simulation data with (A) the anomalous diffusion model or (B) the Ornstein-Uhlenbeck (OU) model of Brownian diffusion under confinement at various value of the ansatz D and of the cell diameter d , normalized by the ansatz D . The grey areas represent $\pm 5\%$ accuracy.

Figure 2 – figure supplement 2 – source data 1. Original values used for plots in Figure 2 – figure supplement 2.

1047

1048 **Figure 2 – figure supplement 3. Comparison between fits of the experimental data with confined diffusion and**
1049 **anomalous diffusion models.** The experimental data (here the example for the R3 measurement from Figure 1 –
1050 figure supplement 2) were fitted by the model of confined diffusion and by the anomalous diffusion model as indicated
1051 by different colors (upper panel), yielding comparable values of residuals (lower panel).

1052

1053 **Figure 4 – figure supplement 1. Effect of osmotic upshift on protein mobility.** The effect of osmotic upshift on
 1054 sfGFP mobility, measured in cells that were either resuspended in tethering buffer (ionic strength of 105 mM) or in
 1055 the same buffer but supplemented with additional 100 mM NaCl (total ionic strength of 305 mM). *** $p < 0.0001$ in a
 1056 two-tailed heteroscedastic t -test.

1057 **Figure 4 – figure supplement 1 – source data 1.** Original values used for plots in Figure 4 – figure supplement 1.

1058

1059 **Figure 4 – figure supplement 2. Effect of growth and measurement temperature on protein diffusion.** Mobility
 1060 for sfGFP in cells grown either at 25°C or at 37°C and measured either at 25°C or at 35°C, as in Figure 4A. ***
 1061 $p < 0.0001$; ** $p < 0.001$; NS: no statistically significant difference in a two-tailed heteroscedastic t -test.

1062 **Figure 4 – figure supplement 2 – source data 1.** Original values used for plots in Figure 4 – figure supplement 2.

1063

1064 **Figure 4 – figure supplement 3. Mass dependence of the effect of antibiotics on protein diffusion.** Data from

1065 Figure 4B were plotted as a function of molecular mass for indicated protein constructs and treatments. Error bars

1066 represent the standard error of the mean.

1067 **Figure 4 – figure supplement 3 – source data 1.** Original values used for plots in Figure 4 – figure supplement 3.

Appendix 1

Notes on the acquisition and analysis protocols for FCS measurements in bacterial cells

Due to the limited size of bacterial cells, FCS measurements require precise positioning of the confocal volume in the bacterial cytoplasm and minimization of the photobleaching-induced effects. In order to ensure that, before fitting an autocorrelation function, we verified the stability of the lateral (xy) positioning of the observation volume by visually analyzing for lateral drifts in confocal images acquired immediately before and after the FCS acquisition. This was done by annotating the xy position in the pre-aquisition image and verifying that the positioning did not change in the post-aquisition image after 120 seconds. Measurements showing xy drift were excluded from the analysis (Appendix 1 - figure 1). Furthermore, the focal stability of the sample was increased by thermal equilibration on the microscope stage before measurements.

Appendix 1 - figure 1. Typical examples of presence or absence of lateral focal drift during FCS measurements.

Substantial lateral drift could be observed for <10% of experiments (upper images), whereas most measurement showed no perceptible lateral drift (lower images).

Long-term photobleaching due to the progressive decrease of the total number of fluorescent proteins during FCS experiments (Appendix 1 - figure 2) is unavoidable due to the small volume of *E. coli* cells, and it requires correction to avoid artifacts. We observed that almost identical ACFs were obtained when correcting for the photobleaching using either multi-segment detrending (Jay Unruh, <https://research.stowers.org/imagejplugins/index.html>, Stowers Institute for Medical Research, USA) or a local averaging approach (Wachsmuth et al. 2015) (Appendix 1 - figure 3).

Appendix 1 - figure 2. Typical traces of fluorescence intensity during FCS measurements. Examples of fluorescence intensity traces for indicated protein fusions. The vertical red dashed lines separate sequential fluorescence intensity acquisitions on the same cell.

Appendix 1 - figure 3. Results of detrending with multi-segments and local averaging approaches. Comparison of experimental ACFs corrected using either multi-segments or local averaging approaches (as indicated) for sfGFP and Adk-sfGFP and different data acquisition segments (R1 vs R6).

We also confirmed that there was no systematic trend in the fitted values of τ_D and α with the time of the fluorescence trace acquisition (Appendix 1 - figure 4). An additional process that could potentially affect autocorrelation functions is short-term photobleaching of the fluorophore in the confocal volume, also known as cryptic photobleaching, which can artificially accelerate the decrease of the autocorrelation function and lead to an underestimation of the protein residence time (Macháň, Foo, and Wohland 2016). This process is different from long-term photobleaching, which is caused by the continuous illumination in the entire illumination light cone. However, the effect of cryptic photobleaching was shown to be typically <5%, even for proteins that diffuse 10 to 100 times slower and have higher bleaching rates than our constructs (Stasevich et al. 2010; Wachsmuth et al. 2015).

43

44

45 **Appendix 1 - figure 4. Values of τ_D or α for the six sequential ACFs.** Values were determined by fitting the
46 anomalous diffusion model to experimental ACFs for the six sequential time segments per individual cell expressing
47 sfGFP (A) or MetH-sfGFP (B).

Appendix 2

Ornstein-Uhlenbeck model for confinement effect in FCS measurements

We aim to derive the autocorrelation function for an FCS experiment in which the fluorescent particles are confined by a (possibly anisotropic) harmonic potential centered at $(x, y, z) = (0, 0, 0)$, i.e.

$$V(x, y, z) = \frac{k_x x^2}{2} + \frac{k_y y^2}{2} + \frac{k_z z^2}{2}$$

where k_i represents the stiffness of the potential in each dimension, and thus the extent σ_i of confinement along that dimension given by $\sigma_i^2 \equiv k_B T / k_i$.

We can treat each dimension independently, using x without loss of generality in what follows. Diffusion in a harmonic potential is described by the Ornstein-Uhlenbeck process. The corresponding Green's function $P(x, t | x_0)$, representing the probability of finding a particle at position x at time t given that it was at position x_0 at time $t = 0$, is

$$P(x, t | x_0) = \frac{1}{\sqrt{2\pi\sigma_x^2 \left(1 - e^{-\frac{2Dt}{\sigma_x^2}}\right)}} \exp \left[-\frac{1}{2\sigma_x^2} \frac{\left(x - x_0 e^{-\frac{Dt}{\sigma_x^2}}\right)^2}{1 - e^{-\frac{2Dt}{\sigma_x^2}}} \right]$$

at long times, $t \rightarrow \infty$, we recover the stationary state given by the Boltzmann distribution corresponding to the harmonic trap

$$P_{\text{st}}(x) = \frac{1}{\sqrt{2\pi\sigma_x^2}} \exp \left[-\frac{x^2}{2\sigma_x^2} \right].$$

The autocorrelation function (ACF) $G(t)$ of an FCS measurement is given as the multiple integral over the product of the probability to detect a photon from a molecule at some initial position x_0 , the probability density that it diffuses from this position to a final position x within time t (given by Green's function), and the probability to detect a photon from a molecule at this final position (Enderlein et al. 2005; Enderlein 2012). Note that the probability of detection of a molecule will necessarily be proportional to the intensity of the laser beam, which we can assume Gaussian and also centered at $(x, y, z) = (0, 0, 0)$, with the usual form

$$I(x, y, z) = I_0 \exp\left(-\frac{2x^2}{\omega_0^2}\right) \exp\left(-\frac{2y^2}{\omega_0^2}\right) \exp\left(-\frac{2z^2}{S^2 \omega_0^2}\right) \equiv I_0 I_x(x) I_y(y) I_z(z)$$

where ω_0 is the width of the (circular) laser beam along the x and y directions, and S is a dimensionless factor accounting for the anisotropy along the z direction, i.e. the axial direction of the beam.

Ignoring constant normalization factors and baselines, the time dependent part of the autocorrelation function is then given by $G(t) = G_x(t)G_y(t)G_z(t)$ with

$$G_x(t) = \int dx \int dx_0 I_x(x) P(x, t | x_0) I_x(x_0) P_{st}(x_0)$$

which can be directly integrated to give, after normalizing so that $G_x(t = 0) = 1$, the expression

$$G_x(t) = \left[1 + 2 \frac{\sigma_x^2}{\omega_0^2} \frac{1 - e^{-\frac{2Dt}{\sigma_x^2}}}{1 + \frac{1}{8} \frac{\omega_0^2}{\sigma_x^2}} \right]^{-\frac{1}{2}}. \quad (\text{A2-1})$$

Note that, in the limit of no confinement, $\sigma_x^2 \rightarrow \infty$, this equation reduces to the well-known ACF for unconfined diffusion

$$G_x(t) = \left[1 + \frac{4Dt}{\omega_0^2} \right]^{-\frac{1}{2}} = \left[1 + \frac{t}{\tau_D} \right]^{-\frac{1}{2}} \quad (A2-1)$$

where we have defined the diffusion time $\tau_D \equiv \omega_0^2/(4D)$. With this definition, the ACF in Eq. (A2-1) can be rewritten as

$$G_x(t) = \left[1 + 2 \frac{\sigma_x^2}{\omega_0^2} \frac{1 - e^{-\frac{1\omega_0^2 t}{2\sigma_x^2 \tau_D}}}{1 + \frac{1}{8} \frac{\omega_0^2}{\sigma_x^2}} \right]^{-\frac{1}{2}}.$$

The full three-dimensional ACF is then, in general,

$$G(t) = \left[1 + 2 \frac{\sigma_x^2}{\omega_0^2} \frac{1 - e^{-\frac{1\omega_0^2 t}{2\sigma_x^2 \tau_D}}}{1 + \frac{1}{8} \frac{\omega_0^2}{\sigma_x^2}} \right]^{-\frac{1}{2}} \left[1 + 2 \frac{\sigma_y^2}{\omega_0^2} \frac{1 - e^{-\frac{1\omega_0^2 t}{2\sigma_y^2 \tau_D}}}{1 + \frac{1}{8} \frac{\omega_0^2}{\sigma_y^2}} \right]^{-\frac{1}{2}} \left[1 + 2 \frac{\sigma_z^2}{S^2 \omega_0^2} \frac{1 - e^{-\frac{1\omega_0^2 t}{2\sigma_z^2 \tau_D}}}{1 + \frac{1}{8} \frac{S^2 \omega_0^2}{\sigma_z^2}} \right]^{-\frac{1}{2}}.$$

The cylindrical geometry of a bacterium can be approximated by an infinite cylinder along the y direction, so that $\sigma_x = \sigma_z = \sigma$ and $\sigma_y \rightarrow \infty$, resulting in the ACF

$$G(t) = \left[1 + 2 \frac{\sigma^2}{\omega_0^2} \frac{1 - e^{-\frac{1\omega_0^2 t}{2\sigma^2 \tau_D}}}{1 + \frac{1}{8} \frac{\omega_0^2}{\sigma^2}} \right]^{-\frac{1}{2}} \left[1 + \frac{t}{\tau_D} \right]^{-\frac{1}{2}} \left[1 + 2 \frac{\sigma^2}{S^2 \omega_0^2} \frac{1 - e^{-\frac{1\omega_0^2 t}{2\sigma^2 \tau_D}}}{1 + \frac{1}{8} \frac{S^2 \omega_0^2}{\sigma^2}} \right]^{-\frac{1}{2}}. \quad (A2-2)$$

Eq. (A2-2), with the added baseline and multiplicative correction accounting for particles in the non-fluorescent state, corresponds to Eq. (3) in the main text.

Appendix 3

Effective diffusion coefficient of two linked proteins

In previous work, we studied the diffusion of two spherical objects with radii a_1 and a_2 , joined together by a flexible linker (Agudo-Canalejo and Golestanian 2020). In the limit of a rigid linker of length ℓ , the effective diffusion coefficient of the composite object goes as:

$$D \simeq \frac{k_B T}{6\pi\eta a_1} \frac{a_1}{(a_1 + a_2)} \left[1 + 2 \frac{a_1 a_2}{(a_1 + a_2)(a_1 + a_2 + \ell)} - \frac{9}{8} \frac{a_1 a_2 (a_1 - a_2)^2}{(a_1 + a_2)^2 (a_1 + a_2 + \ell)^2} \right] \quad (\text{A3-1})$$

plus higher order correction terms of order $O\left(\frac{a_i^3}{(a_1 + a_2 + \ell)^3}\right)$.

We can then consider what is the effective diffusion coefficient of two proteins that are linked to each other. For that, we first need to connect the molecular mass to the effective radius of the protein. If we identify subunit 1 with GFP, and subunit 2 with the protein attached to it, and we call M_{GFP} the molecular mass of GFP and M_{tot} the total molecular mass (sum of GFP and the protein), we expect relations of the form

$$a_1 = C M_{\text{GFP}}^\beta$$

$$a_2 = C (M_{\text{tot}} - M_{\text{GFP}})^\beta$$

where C is a proportionality constant assumed to be typical for all proteins (which is of order 1, with values reported in the literature of about 0.65 (Smilgies and Foltá-Stogniew 2015), when the mass is in kDa and the radius is in nm), and β is the scaling exponent introduced in the main text. For the linker which is made of 6 amino-acids, we may use the typical conversion factor 0.35 nm/amino-acid to estimate $\ell \approx 2$ nm.

113 Plugging these expressions for a_1 and a_2 into Eq. (A3-1) above, one obtains an expression for the
 114 diffusion coefficient D as a function of M_{tot} that depends only on three parameters (since the
 115 molecular mass of GFP is known): (i) the diffusion coefficient of GFP $\frac{k_B T}{6\pi\eta a_1}$, (ii) the exponent β ,
 116 and (iii) the rescaled linker length ℓ/C .

Appendix 4

Exact p-values for all significance analyses

Figure 1C

<i>Testing pair</i>	<i>P-value</i>
sfGFP vs Adk-sfGFP	0.000000010
Adk-sfGFP vs AcnA-sfGFP	0.00000000044

Figure 1 – figure supplement 6A

<i>Testing pair</i>	<i>P-value</i>
ClpS ^{WT} -sfGFP vs ClpS ^{D35A_D36A_H66A} -sfGFP	0.000094
Map ^{WT} -sfGFP vs Map ^{Lys→Ala} -sfGFP	0.0000012
Map ^{WT} -sfGFP vs Map ^{Lys→Glu} -sfGFP	0.000000016
Map ^{Lys→Ala} -sfGFP vs Map ^{Lys→Glu} -sfGFP	0.10
DnaK ^{WT} -sfGFP vs DnaK ^{V436F} -sfGFP	0.00023

Figure 1 – figure supplement 6B

<i>Testing pair</i>	<i>P-value</i>
ClpS ^{WT} -sfGFP vs ClpS ^{D35A_D36A_H66A} -sfGFP	0.000014
Map ^{WT} -sfGFP vs Map ^{Lys→Ala} -sfGFP	0.0092
Map ^{WT} -sfGFP vs Map ^{Lys→Glu} -sfGFP	0.065
Map ^{Lys→Ala} -sfGFP vs Map ^{Lys→Glu} -sfGFP	0.37
DnaK ^{WT} -sfGFP vs DnaK ^{V436F} -sfGFP	0.00000019

Figure 1 – figure supplement 7A

<i>Testing pair</i>	<i>P-value</i>
Adk ^{E.c.} -sfGFP vs Adk ^{Y.c.} -sfGFP	0.17
Adk ^{E.c.} -sfGFP vs Adk ^{V.c.} -sfGFP	0.056
Adk ^{E.c.} -sfGFP vs Adk ^{C.c.} -sfGFP	0.93
Adk ^{E.c.} -sfGFP vs Adk ^{M.x.} -sfGFP	0.21
Adk ^{E.c.} -sfGFP vs Adk ^{B.s.} -sfGFP	0.23
Pgk ^{E.c.} -sfGFP vs Pgk ^{V.c.} -sfGFP	0.75
Pgk ^{E.c.} -sfGFP vs Pgk ^{C.c.} -sfGFP	0.26
Pgk ^{E.c.} -sfGFP vs Pgk ^{M.x.} -sfGFP	0.33
AcnA ^{E.c.} -sfGFP vs AcnA ^{Y.c.} -sfGFP	0.093
AcnA ^{E.c.} -sfGFP vs AcnA ^{V.c.} -sfGFP	0.084
AcnA ^{E.c.} -sfGFP vs AcnA ^{M.x.} -sfGFP	0.0000000023
AcnA ^{E.c.} -sfGFP vs AcnA ^{B.s.} -sfGFP	0.069

Figure 1 – figure supplement 7B

<i>Testing pair</i>	<i>P-value</i>
Adk ^{E.c.} -sfGFP vs Adk ^{Y.c.} -sfGFP	0.000060
Adk ^{E.c.} -sfGFP vs Adk ^{V.c.} -sfGFP	0.18
Adk ^{E.c.} -sfGFP vs Adk ^{C.c.} -sfGFP	0.042
Adk ^{E.c.} -sfGFP vs Adk ^{M.x.} -sfGFP	0.070
Adk ^{E.c.} -sfGFP vs Adk ^{B.s.} -sfGFP	0.0029
Pgk ^{E.c.} -sfGFP vs Pgk ^{V.c.} -sfGFP	0.11
Pgk ^{E.c.} -sfGFP vs Pgk ^{C.c.} -sfGFP	0.0082
Pgk ^{E.c.} -sfGFP vs Pgk ^{M.x.} -sfGFP	0.0087
AcnA ^{E.c.} -sfGFP vs AcnA ^{Y.c.} -sfGFP	0.035
AcnA ^{E.c.} -sfGFP vs AcnA ^{V.c.} -sfGFP	0.16
AcnA ^{E.c.} -sfGFP vs AcnA ^{M.x.} -sfGFP	0.083
AcnA ^{E.c.} -sfGFP vs AcnA ^{B.s.} -sfGFP	0.00024

Figure 2C

<i>Testing pair</i>	<i>P-value</i>
Untreated vs A22 treatment	0.00000000000032

Figure 2D

<i>Testing pair</i>	<i>P-value</i>
Untreated vs A22 treatment	0.0022

Figure 4A

<i>Testing pair</i>	<i>P-value</i>
sfGFP 25°C vs 35°C	0.000040
Adk-sfGFP 25°C vs 35°C	0.0000025
AcnA-sfGFP 25°C vs 35°C	0.00077

Figure 4B

<i>Testing pair</i>	<i>P-value</i>
sfGFP Untreated vs Chloramphenicol	0.61
sfGFP DMSO vs Rifampicin	0.012
Adk-sfGFP Untreated vs Chloramphenicol	0.12
Adk-sfGFP DMSO vs Rifampicin	0.00048
Pgk-sfGFP Untreated vs Chloramphenicol	0.17
Pgk-sfGFP DMSO vs Rifampicin	0.0000012
AcnA-sfGFP Untreated vs Chloramphenicol	0.000011
AcnA-sfGFP DMSO vs Rifampicin	0.0085

Figure 4 – figure supplement 1

<i>Testing pair</i>	<i>P-value</i>
sfGFP ionic strength 105 mM vs 305 mM	0.000027

Figure 4 – figure supplement 2

<i>Testing pair</i>	<i>P-value</i>
sfGFP Grown 25°C, measured 25°C vs 35°C	0.0020
sfGFP Measured 25°C, grown 25°C vs 37°C	0.060
sfGFP Measured 35°C, grown 25°C vs 37°C	0.98
sfGFP Grown 37°C, measured 25°C vs 35°C	0.000040

Figure 5

<i>Testing pair</i>	<i>P-value</i>
sfGFP M9 salts vs M9 salts, Cam	0.82
sfGFP M9 salts vs M9 salts, Glu+CA	0.000023
sfGFP M9 salts, Cam vs M9 salts, Cam, Glu+CA	0.019
sfGFP M9 salts, Glu+CA vs M9 salts, Cam, Glu+CA	0.019
AcnA-sfGFP M9 salts vs M9 salts, Cam	0.37
AcnA-sfGFP M9 salts vs M9 salts, Glu+CA	0.0073
AcnA-sfGFP M9 salts, Cam vs M9 salts, Cam, Glu+CA	0.0035
AcnA-sfGFP M9 salts, Glu+CA vs M9 salts, Cam, Glu+CA	0.014

Appendix 5

Numerosity of the constructs and conditions for each experiment

Table 1. Figure 1 and Figure 1 – figure supplement 4 and 6

<i>Construct</i>	<i>Numerosity (n)</i>	<i>Construct</i>	<i>Numerosity (n)</i>
sfGFP	42	Pgk-sfGFP	16
YggX-sfGFP	8	EntC-sfGFP	15
ClpS ^{WT} -sfGFP	11	AroA-sfGFP	9
ClpS ^{D35A_D36A_H66A} -sfGFP	10	ThrC-sfGFP	14
FolK-sfGFP	8	MurF-sfGFP	7
Crr-sfGFP	14	DsdA-sfGFP	14
UbiC-sfGFP	14	HemN-sfGFP	13
CoaE-sfGFP	11	PrpD-sfGFP	12
Adk-sfGFP	23	DnaK ^{WT} -sfGFP	10
Cmk-sfGFP	16	DnaK ^{V436F} -sfGFP	10
KdsB-sfGFP	22	MalZ-sfGFP	9
Map ^{WT} -sfGFP	20	GlcB-sfGFP	16
Map ^{Lys→Glu} -sfGFP	10	MetE-sfGFP	8
Map ^{Lys→Ala} -sfGFP	12	LeuS-sfGFP	14
MmuM-sfGFP	14	AcnA-sfGFP	19
PanE-sfGFP	18	MetH-sfGFP	9
SolA-sfGFP	7		

Table 2. Figure 1 – figure supplement 7

<i>Construct</i>	<i>Numerosity (n)</i>	<i>Construct</i>	<i>Numerosity (n)</i>
Adk ^{E.c.} -sfGFP	Same as table 1	Pgk ^{C.c.} -sfGFP	5
Adk ^{Y.c.} -sfGFP	5	Pgk ^{M.x.} -sfGFP	11
Adk ^{V.c.} -sfGFP	10	AcnA ^{E.c.} -sfGFP	Same as table 1
Adk ^{C.c.} -sfGFP	5	AcnA ^{Y.c.} -sfGFP	5
Adk ^{M.x.} -sfGFP	11	AcnA ^{V.c.} -sfGFP	10
Adk ^{B.s.} -sfGFP	10	AcnA ^{M.x.} -sfGFP	10
Pgk ^{E.c.} -sfGFP	Same as table 1	AcnA ^{B.s.} -sfGFP	10
Pgk ^{V.c.} -sfGFP	10		

Table 3. Figure 2C and 2D

<i>Condition</i>	<i>Numerosity (n)</i>
Untreated	10
A22-treated	12

Table 4. Figure 3

<i>Construct</i>	<i>Numerosity (n)</i>	<i>Construct</i>	<i>Numerosity (n)</i>
sfGFP	11	DsdA-sfGFP	10
YggX-sfGFP	10	GlcB-sfGFP	10
Adk-sfGFP	16	AcnA-sfGFP	10
PanE-sfGFP	11	MetH-sfGFP	15

Table 5. Figure 4A

<i>Construct and condition</i>	<i>Numerosity (n)</i>	<i>Construct and condition</i>	<i>Numerosity (n)</i>
sfGFP, 25°C	See table 1	AcnA-sfGFP, 25°C	See table 1
sfGFP, 35°C	14	AcnA-sfGFP, 35°C	18
Adk-sfGFP, 25°C	See table 1		
Adk-sfGFP, 35°C	21		

Table 5. Figure 4B

<i>Construct and condition</i>	<i>Numerosity (n)</i>	<i>Construct and condition</i>	<i>Numerosity (n)</i>
sfGFP, untreated	See table 1	Pgk-sfGFP, untreated	See table 1
sfGFP, chloramphenicol	10	Pgk-sfGFP, chloramphenicol	10
sfGFP, DMSO	15	Pgk-sfGFP, DMSO	10
sfGFP, rifampicin	15	Pgk-sfGFP, rifampicin	10
Adk-sfGFP, untreated	See table 1	AcnA-sfGFP, untreated	See table 1
Adk-sfGFP, chloramphenicol	10	AcnA-sfGFP, chloramphenicol	10
Adk-sfGFP, DMSO	10	AcnA-sfGFP, DMSO	10
Adk-sfGFP, rifampicin	10	AcnA-sfGFP, rifampicin	10

Table 6. Figure 4 – figure supplement 1

<i>Construct and condition</i>	<i>Numerosity (n)</i>
sfGFP, 105 mM ionic strength	See table 1
sfGFP, 305 mM ionic strength	11

Table 6. Figure 4 – figure supplement 2

<i>Construct and condition</i>	<i>Numerosity (n)</i>
sfGFP, grown 25°C, measured 25°C	10
sfGFP, grown 25°C, measured 35°C	10
sfGFP, grown 37°C, measured 25°C	See table 1
sfGFP, grown 37°C, measured 35°C	See table 5

Table 7. Figure 5

<i>Construct and condition</i>	<i>Numerosity (n)</i>	<i>Construct and condition</i>	<i>Numerosity (n)</i>
sfGFP, M9 salts	10	AcnA-sfGFP, M9 salts	5
sfGFP, M9 salts, Cam	13	AcnA-sfGFP, M9 salts, Cam	8
sfGFP, M9 salts, Glu+CA	15	AcnA-sfGFP, M9 salts, Glu+CA	10
sfGFP, M9 salts, Cam, Glu+CA	13	AcnA-sfGFP, M9 salts, Cam, Glu+CA	10