Supplementary Information

Super-resolving microscopy reveals the localizations and movement dynamics of stressosome proteins in *Listeria monocytogenes*

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Keywords: *Listeria monocytogenes,* protein localization, protein diffusion, Prli42, RsbR1, RsbL, stressosome, super-resolution microscopy, single-particle tracking, single-molecule displacement mapping

Table 1. Molecular weight (M_W), pI, and net charge of proteins of interest

Protein/Fusion	Theoretical M_W (kDa)	Average_pI	Average net charge at pH 7.5
mEos3.2	26.2	7.33	-0.49
mEos3.2-prli42	30.1	8.14	1.49
mEos3.2-RsbR1	57.8	5.50	-14.53
mEos3.2-RsbL	55.2	5.29	-21.46
RsbR1	31.6	4.70	-14.28
RsbL	28.8	4.60	-20.21

Protein sequences

>RsbR1 (genome) || Molecular weight: 31620.5 Da

MYKDFANFIRTNKADLLNDWMNEMEKQSDQLINDIAKEAMYEETSKEFVDLIVSNVTENGS
KFNEKLDDFAEKVVHLGWPIHFVTTGLRVFGLLVYTAMRDEDLFLKREEKPEDDAYYRFET
WLSSMYNKVVTAYADTWEKTVSIQKSALQELSAPLLPIFEKISVMPLIGTIDTERAKLIIENLLI
GVVKNRSEVVLIDITGVPVVDTMVAHHIIQASEAVRLVGCQAMLVGIRPEIAQTIVNLGIELD
QIITTNTMKKGMERALALTNREIVEKEG

>RsbL (genome) || Molecular weight: 28816.77 Da

MTAYPQFDVILKALNLSSVGVIITDPEQKDNPIIFVNTGFENITGYAKEEALGSNCHFLQGDDT
DKEEVAKIRHAINEKSTANVLLKNYRKDGTSFMNELTIEPIYDDHEHLYFVGIQKDVTTEHD
YQLELEKSLTEIEKLSTPIVPIKENICVLPLIGSLTHDRFQHMSEYVSEYMDHGKEDYLIMDLS
GLAEFNEDAVMNLVKFHGFMKLTGVELIITGISPKFAMTLIRYEENLASLTTYSTIKEALQFY

>mEos3.2 (in pNF mEos3.2) || Molecular weight: 26217.64 Da

MGSAIKPDMKIKLRMEGNVNGHHFVIDGDGTGKPFEGKQSMDLEVKEGGPLPFAFDILTTAF
HYGNRVFAKYPDNIQDYFKQSFPKGYSWERSLTFEDGGICNARNDITMEGDTFYNKVRFYG
TNFPANGPVMQKKTLKWEPSTEKMYVRDGVLTGDIEMALLLEGNAHYRCDFRTTYKAKEK
GVKLPGAHFVDHCIEILSHDKDYNKVKLYEHAVAHSGLPDNARR**GGTGGS**

>mEos3.2-Prli42 (in pNF mEos3.2-prli42) || Molecular weight: 30098.58 Da

MGSAIKPDMKIKLRMEGNVNGHHFVIDGDGTGKPFEGKQSMDLEVKEGGPLPFAFDILTTAF
HYGNRVFAKYPDNIQDYFKQSFPKGYSWERSLTFEDGGICNARNDITMEGDTFYNKVRFYG
TNFPANGPVMQKKTLKWEPSTEKMYVRDGVLTGDIEMALLLEGNAHYRCDFRTTYKAKEK
GVKLPGAHFVDHCIEILSHDKDYNKVKLYEHAVAHSGLPDNARR**GGTGGS**PWMTNKKVVR
VVVILMLIAIVLSSVLTGVLMFLVD

>mEos3.2-RsbR1 (in pNF mEos3.2-RsbR1) || Molecular weight: 57833.06 Da

MGSAIKPDMKIKLRMEGNVNGHHFVIDGDGTGKPFEGKQSMDLEVKEGGPLPFAFDILTTAF
HYGNRVFAKYPDNIQDYFKQSFPKGYSWERSLTFEDGGICNARNDITMEGDTFYNKVRFYG
TNFPANGPVMQKKTLKWEPSTEKMYVRDGVLTGDIEMALLLEGNAHYRCDFRTTYKAKEK
GVKLPGAHFVDHCIEILSHDKDYNKVKLYEHAVAHSGLPDNARR**GGTGGS**GSYKDFANFIR
TNKADLLNDWMNEMEKQSDQLINDIAKEAMYEETSKEFVDLIVSNVTENGSKFNEKLDDFA
EKVVHLGWPIHFVTTGLRVFGLLVYTAMRDEDLFLKREEKPEDDAYYRFETWLSSMYNKV
VTAYADTWEKTVSIQKSALQELSAPLLPIFEKISVMPLIGTIDTERAKLIIENLLIGVVKNRSEV
VLIDITGVPVVDTMVAHHIIQASEAVRLVGCQAMLVGIRPEIAQTIVNLGIELDQIITTNTMKK
GMERALALTNREIVEKEG

>mEos3.2-RsbL (in pNF mEos3.2-RsbL) || Molecular weight: 55161.5 Da

MGSAIKPDMKIKLRMEGNVNGHHFVIDGDGTGKPFEGKQSMDLEVKEGGPLPFAFDILTTAF
HYGNRVFAKYPDNIQDYFKQSFPKGYSWERSLTFEDGGICNARNDITMEGDTFYNKVRFYG
TNFPANGPVMQKKTLKWEPSTEKMYVRDGVLTGDIEMALLLEGNAHYRCDFRTTYKAKEK
GVKLPGAHFVDHCIEILSHDKDYNKVKLYEHAVAHSGLPDNARR**GGTGGS**EFTAYPQFDVI
LKALNLSSVGVIITDPEQKDNPIIFVNTGFENITGYAKEEALGSNCHFLQGDDTDKEEVAKIRH
AINEKSTANVLLKNYRKDGTSFMNELTIEPIYDDHEHLYFVGIQKDVTTEHDYQLELEKSLTE
IEKLSTPIVPIKENICVLPLIGSLTHDRFQHMSEYVSEYMDHGKEDYLIMDLSGLAEFNEDAV
MNLVKFHGFMKLTGVELIITGISPKFAMTLIRYEENLASLTTYSTIKEALQFY

RsbR1: Blue sequence/ RsbL: Dark golden sequence/ mEos3.2: Green sequence/ Prli42: Magenta sequence/ **GGTGGS:** hexa-amino acid linker

67 **Table 2. List of strains and plasmids used in this paper**

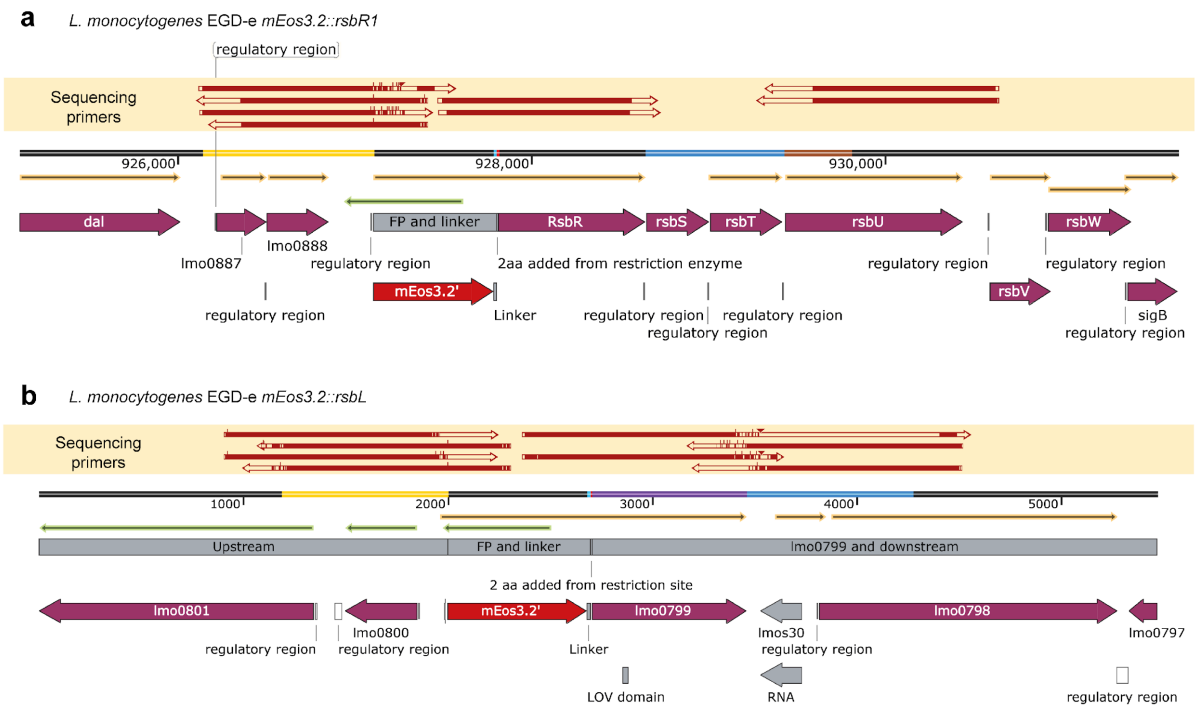
Bacteria strains	Reference/source
Escherichia coli	
K12	NovaBlue
MC1061 *ΔarcB* (Erythromycin sensitive)	[1]
Listeria monocytogenes	[2]
EGD-e	K. Boor (Connor O'Byrne's lab)
EGD-e *ΔrsbR1*	[2]
EGD-e *mEos3.2::rsbR1*	This study
EGD-e *ΔrsbL*	[3]
EGD-e *mEos3.2::rsbL*	This study
EGD-e *ΔsigB*	[4]
EGD-e *Δprli42*	[5]
Plasmid	
pMAD	[6]
pMAD mEos3.2-RsbR1	This study
pMAD mEos3.2-RsbL	This study
pNF GFP-RsbR1	This study
pNF mEos3.2	This study
pNF mEos3.2-Prli42	This study
pNF mEos3.2-RsbR1 (including in-frame RsbS and RsbT))	This study
pNF mEos3.2-RsbL	This study
pNF RsbL	This study

68

69 **Table 3. List of primers**

No.	Name	Function	Sequence (5' □ 3')	Reference
1.	Fw Up_RsbL	Forward primer (carries NcoI site) and reverse primer (carries SalI site) to amplify the 811 bp sequence upstream of the gene *rsbL* (*lmo0799*)	CCACCATGGCAGGATCTGG AAAAAGATC	This study
2.	Rv Up_RsbL		GTCGTCGACCTGCGTGTTTC TCCCCCT	This study
3.	Fw mEos3.2 (L)	Forward primer (carries SalI site) and reverse primer (carries EcoRI site) to amplify the gene *mEos3.2* with a 6-amino acid linker	GTCGTCGACATGGGAAGTG CGATTAAGCC	This study
4.	Rv mEos3.2 (L)		GAAGAATTCCGAACCGCCG GTACCGCCTCGTCTGGCATT GTCAGG	This study
5.	Fw RsbL	Forward primer (carries EcoRI site) and reverse primer (carries NcoI site) to amplify the gene *rsbL (lmo0799)* and its 811 bp sequence downstream	GAAGAATTCACCGCTTATCC ACAATTCGATG	This study
6.	Rv RsbL		CCACCATGGTTCTGATTCTC CGTAAGCTTTTACC	This study
7.	Fw Up_RsbR1	Forward primer (carries NcoI site) and reverse primer (carries SalI site) to amplify the 964 bp sequence upstream of the gene *rsbR1(lmo0889)*	CCACCATGGGCGAAATAAC TCTTTTCTTCAAGGC	This study
8.	Rv Up_RsbR1		GTCGTCGACCAGTTATTTTC CAACCTTTCTCCAC	This study
9.	Rv mEos3.2 (1)	Reverse primer (carries BamHI site) to amplify the gene *mEos3.2* with 6-amino acid linker; used with the forward primer of Fw mEos3.2 (L)	GGAGGATCCCGAACCGCCG GTACCGCCTCGTCTGGCATT GTCAGG	This study
10.	Fw RsbR1	Forward primer (carries BamHI site) and reverse primer (carries NcoI site) to amplify the gene *rsbR1 (lmo0889*) and 1160 bp sequence downstream, including the genes *rsbS*, *rsbT,* and part of *rsbU*	GGAGGATCCTATAAAGATTT TGCAAACTTCATCCG	This study
11.	Rv RsbR1_short		CCACCATGGCCCAAAATCA AGTTCATCGTG	This study
12.	Fw upup_RsbL	Forward primer at 1126 bp upstream of the gene *rsbL (lmo0799*) to confirm the chromosomal integration	GGTTATTTCCGCAATAGAAA GTAG	This study
13.	Rv mid_mEos3.2	Reverse primer in the middle of the gene *mEos3.2* to confirm chromosomal integration	CCTTCCATTGTTATGTCGTTT C	This study
14.	Fw mid_mEos3.2	Forward primer in the middle of the gene *mEos3.2* to confirm the chromosomal integration	GGACACTTTCTATAATAAAG TTCGATT	This study
15.	Rv downdown_Rsb L	Reverse primer at 1081 bp downstream of gene *rsbL (lmo0799*) to confirm the chromosomal integration	CCAGAACACTTGTTTAATCG C	This study
16.	Fw upup_RsbR1	Forward primer at 1015 bp upstream of the gene *rsbR1 (lmo0889)* to confirm the chromosomal integration	GGACTTAAGGGAGTATATC AGCT	This study
17.	Rv downdown_Rsb R1	Reverse primer at 2029 bp downstream of the gene *rsbR1 (lmo0889)* to confirm the chromosomal integration	GCTGTATAAGCATCGATCTC C	This study
18.	Rv mEosRST	Reverse primer to clone RsbRST from pMAD mEos3.2-RsbR1; carries NcoI site	GCCGCCATGGTTACCGAACC CATTTCG	This study
19.	Fw pNF	Forward primer to clone the pNF8 backbone creating pNF mEos3.2-RsbRST; carries NcoI site	CCACCATGGCTGCAGGCATG CAAGC	This study
20.	Rv pNF	Reverse primer to clone pNF8 backbone creating pNF mEos3.2-RsbRST; carries SalI site	GTCGTCGACTTAATAAACCT CCTTTCGGATCCGTTG	This study
21.	Rv mEosRsbL/ RsbL(pNF)	Reverse primer to clone mEos3.2-RsbRL or rsbL from pMAD mEos3.2-RsbL; carries NcoI site	CCACCATGGTTAGTAAAATT GTAATGCTTCTTTGATTGTG C	This study
22.	Fw RsbRL(pNF)	Forward primer to clone *rsbL* (*lmo0799*) from pMAD mEos3.2-RsbL; carries SalI site	GTCGTCGACATGACCGCTTA TCCACAATTCG	This study
23.	Fw mEos3.2 pNF8	Forward primer to clone gene for mEos3.2 from pMAD mEos3.2-RsbR1	AGGTTTATTAAAUGGGAAG TGCGATTAAGC	This study
24.	Rv mEos3.2 pNF8	Reverse primer to clone gene for mEos3.2 from pMAD mEos3.2-RsbR1	AGTTATTAUGAACCGCCGGT ACCG	This study

25.	Fw pNF8 (prli42)	Forward primer to open pNF8 backbone	ATAATAACUGCAGGCATGC AAGCTTG	This study
26.	Rv pNF8 mEos3.2	Reverse primer to open pNF8 backbone	ATTTAATAAACCUCCTTTCG GATCCGTTG	This study
27.	Fw-pNF8 sequencing	Forward primer for sequencing to check for proper insertion of mEos3.2	TGAGCGCAACGCAATTAAT G	This study
28.	Rv-pNF8 sequencing	Reverse primer for sequencing to check for proper insertion of mEos3.2	CGATTAAGTTGGGTAACGCC AG	This study
29.	Fw prli42 (res)	Forward primer to amplifying *prli42* from pAD prli42; carries NcoI site	CCACCATGGATGACTAATAA AAAAGTAGTTCGC	This study
30.	Rv prli42 (res)	Reverse primer to amplifying *prli42* from pAD prli42; carries SalI site	GTCGTCGACTAAAAACATTA ATACCCCGGTT	This study
31.	Fw pNFmEos (res)	Forward primer to amplifying pNF mEos3.2; carries SalI site	GTCGTCGACTAATAACTGCA GGCATGCAAGC	This study
32.	Rv pNFmEos (res)	Reverse primer to amplifying pNF mEos3.2; carries NcoI site	CCACCATGGTGAACCGCCG GTACCG	This study



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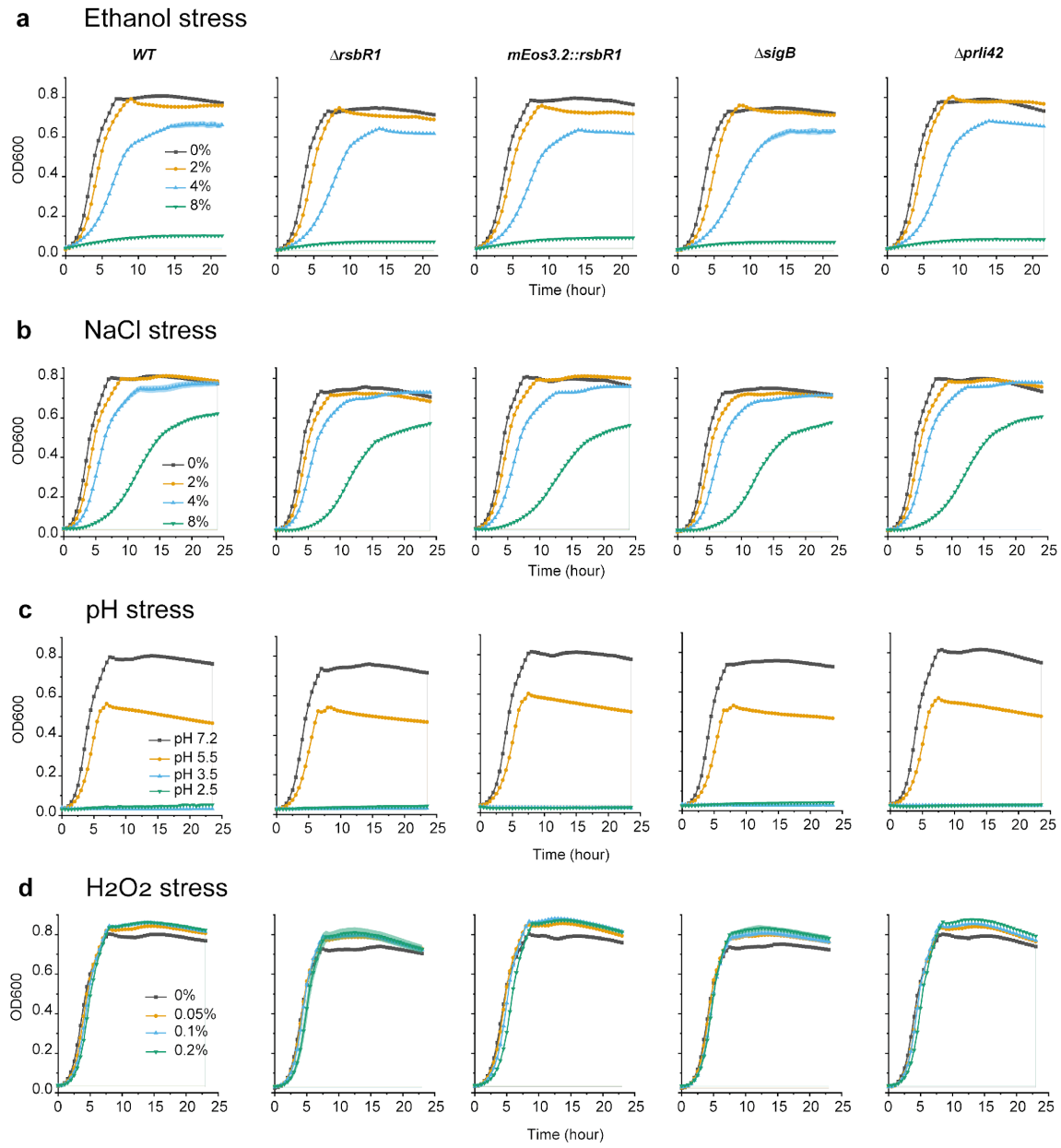
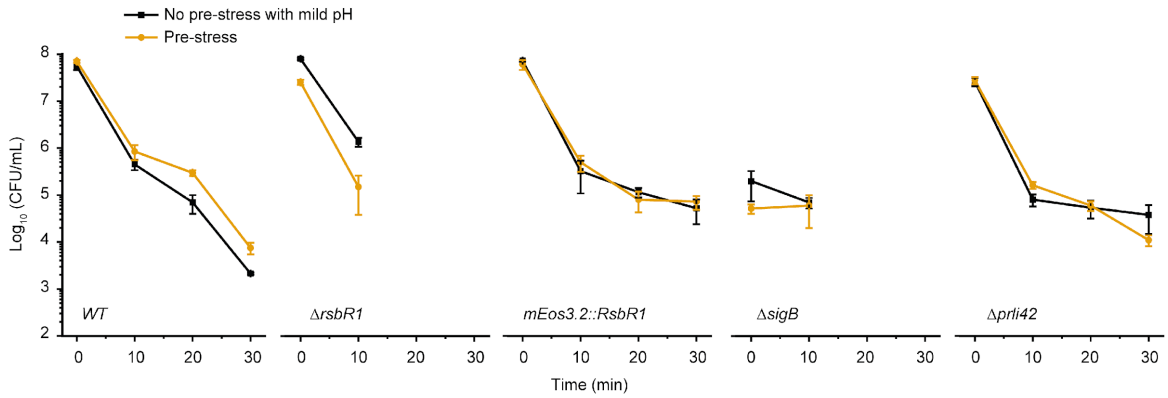
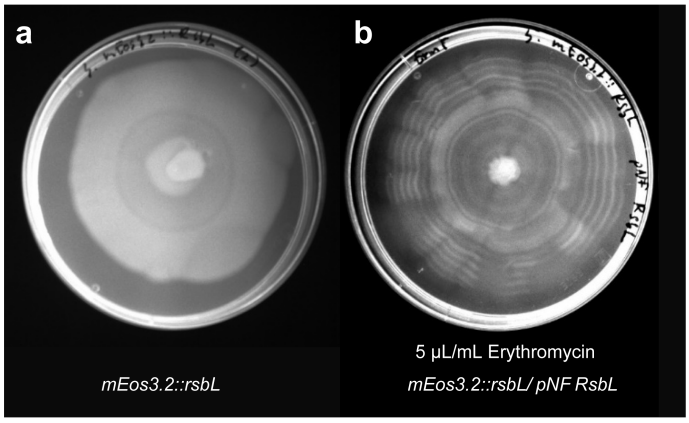
73 **Supplementary Fig. 1 | Sequencing results of the integration strains. a**, and **b**, *L.*
74 *monocytogenes* EGD-e *mEos3.2::rsbR1* and *L. monocytogenes* EGD-e *mEos3.2::rsbL*,
75 respectively. Two colonies of each integrant were sequenced. After chromosomal
76 integration of *mEos3.2::rsbR1* and *mEos3.2::rsbL*, the plasmids were excised as shown by X-
77 gal (blue-white) and antibiotic sensitivity screens. Selected colonies were checked for
78 integration by colony-PCR and sequencing with primers upstream, downstream, and two
79 middle positions of the gene *mEos3.2*.



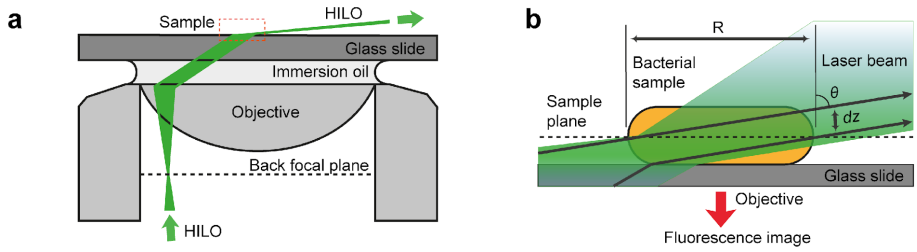
Supplementary Fig. 2 | Growth under ethanol, NaCl, low pH, and H_2O_2 stress of *L. monocytogenes* wild-type, *ΔrsbR1,* integrant *mEos3.2::rsbR1, ΔsigB,* and *Δprli42*. The growth was monitored in 96-well plates at 30 ^{o}C with 200 rpm shaking. Cells were grown in BHI medium supplemented with **(a)** ethanol at 0% (control), 2%, 4%, and 8% (v.v^{-1}); **(b)** NaCl at 0% (control), 2%, 4%, and 8% (w.v^{-1}); **(c)** HCl to set the pH to pH 7.2 (control), pH 5.5, pH 3.5, and pH 2.5; and **(d)** with H_2O_2 at 0% (control), 0.05%, 0.1% and 0.2%.



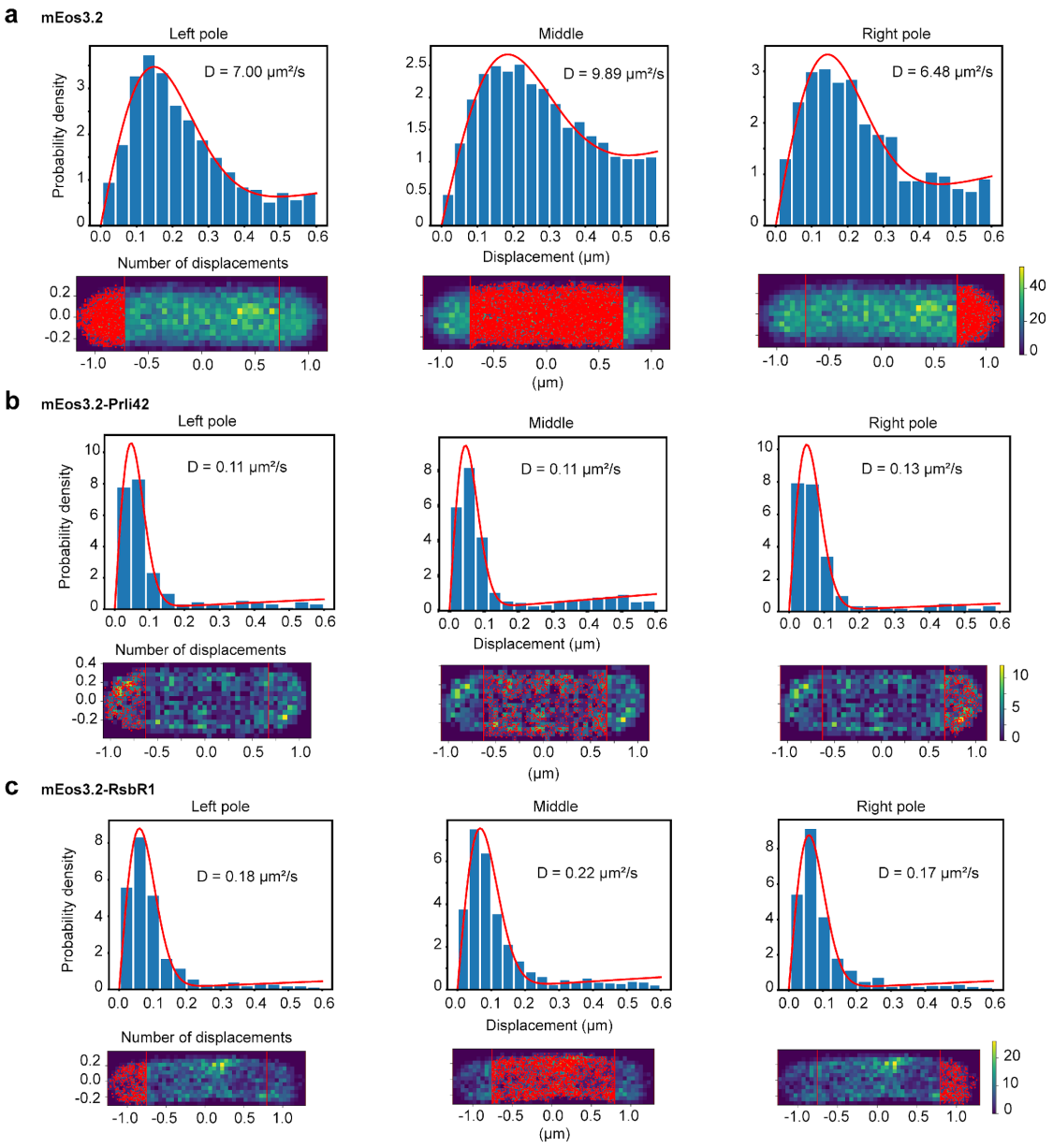
Supplementary Fig. 3 | Phenotypic test of survival of *L. monocytogenes* upon acid shock treatment. Mid-log phase cultures untreated at pH 7.2 (No pre-stress) or treated at pH 5.0 (Pre-stress) for 15 min and subsequently challenged in acidified BHI (pH 2.5). The detection threshold is 10^2 CFU.mL^{-1}. Samples were taken at 0, 10, 20, and 30 min. Survival data are expressed as Log_{10} (CFU.mL^{-1}). *ΔrsbR1* and *ΔsigB* were notably more sensitive to the acid treatment than wild-type, *mEos3.2::rsbR1*, and *Δprli42* and colonies were no longer detected after 10 min treatment at pH 2.5. Error bars represent standard deviations. Three biological replicates were made.



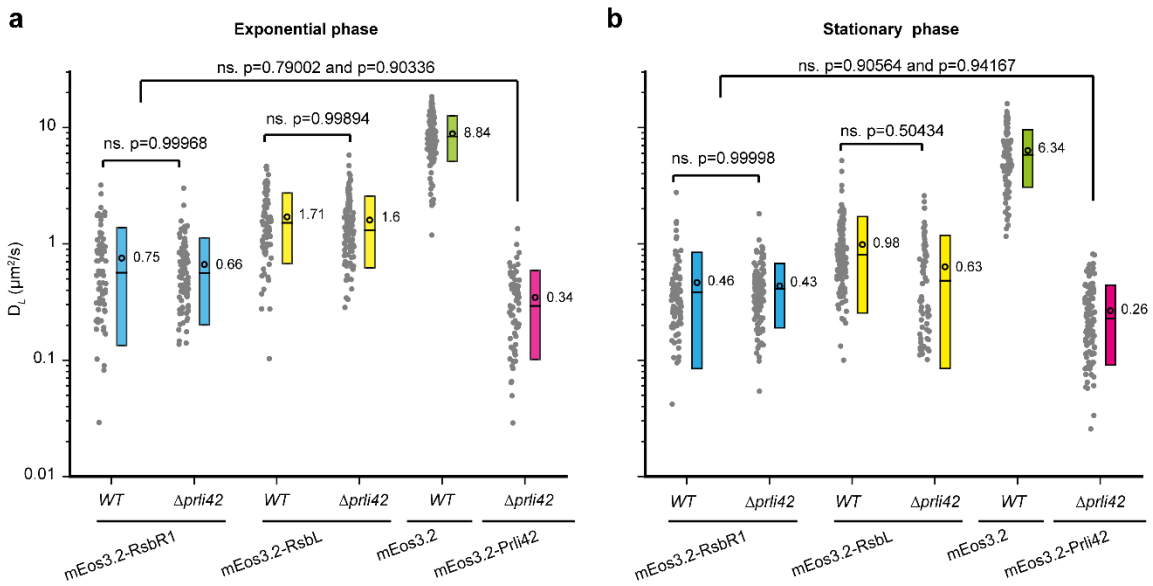
Supplementary Fig. 4 | Ring formation phenotype on low-agar after twelve oscillating cycles of light and dark (12-hour intervals). a, and **b,** The integration strain *mEos3.2::rsbRL* and the same strain expressing *rsbL* from the P_{dlt} promoter of pNF RsbL. We used an erythromycin concentration for plasmid selection of 5 µg.mL^{-1}. Images are representative of at least three independent experiments.



Supplementary Fig. 5 | Schematic of the highly inclined and laminated optical sheet (HILO) microscopy, adapted from the previous study[7]. **a**, The set-up of optics. **b**, Bacterial sample is illuminated with a laminated thin sheet of the laser beam. The *dz* is the thickness of the geometrical optics along the z-direction and $dz = R/\tan\theta$, where *R* is the diameter of the illuminated area, and θ is the incidence angle at the sample.



Supplementary Fig. 6 | Diffusion at subcellular regions. a, **b**, and **c**, show the subcellular division of free mEos3.2, mEos3.2-Prli42, and mEos3.2-RsbR1, respectively. Cells were divided into three main regions: the cell center (60% in length) and the cell poles (each 20% in length). The displacements belonging to the corresponding regions were then analyzed separately. The top panels are the distributions of all single-molecule displacements from the corresponding regions shown in the bottom panels. The red curve is obtained from MLE fitting, using equation (4), and the resulting diffusion coefficients are presented inside the panels.



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121 **Supplementary Fig. 7 | Ensemble diffusion coefficients determined by FRAP**
122 **measurements**. **a,** and **b,** Box charts of the D_L from FRAP measurements showing the
123 ensemble mobility of mEos3.2-RsbR1, mEos3.2-RsbL, mEos3.2-Prli42, and mEos3.2 in the
124 wild-type and *⊿prli42* strains in the exponential and stationary phase of growth. Each dot
125 shows the data of one cell. The box range indicates the standard deviation (SD), and the
126 open circle and dashed symbols inside the boxes indicate the mean and median,
127 respectively. (ns) is not significant and statistical significance was determined by one-way
128 ANOVA followed by Tukey's post-hoc test to calculate P-values.

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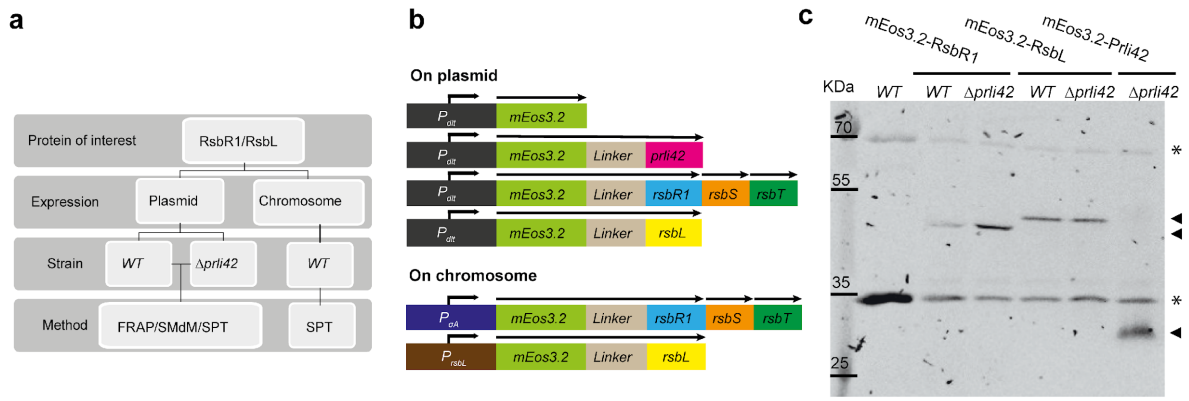

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131 **Supplementary Fig. 8 | Experimental overview, protein tagging strategy, electrophoretic**
132 **mobility, and in-gel fluorescence of proteins. a**, Experimental overview for measuring the
133 intracellular dynamics of stressosome proteins in live cells. The targeted proteins are
134 RsbR1, and RsbL produced either from a multi-copy plasmid or the chromosome in wild-
135 type (WT) or *⊿prli42* strains. The diffusion coefficients of the proteins are measured by
136 FRAP, SMdM, or SPT. **b**, A photoconvertible fluorescent protein mEos3.2 is fused to the N-
137 terminus of mEos3.2-RsbR1 and mEos3.2-RsbL, and the fusion proteins are produced from
138 a plasmid or the chromosome. Free cytosolic mEos3.2 and mEos3.2 fused to the N-
139 terminus of Prli42 (mEos3.2-Prli42) are produced from pNF mEos3.2-Prli42, which is under
140 the control of the constitutive P_{dlt} promoter; while the chromosomal expression is driven
141 by corresponding native promoters, which are P_{sigA} and P_{rsbL} for *rsbR1* and *rsbL*,
142 respectively. **c**, In-gel fluorescence of the constructs tagged with mEos3.2 produced in the
143 wild-type and *⊿prli42* strains. The expected bands are shown by black triangle symbols. A
144 star symbol represents endogenous fluorescence in *L. monocytogenes*. The molecular
145 weight (kDa) of the marker proteins is indicated on the left of the panel. These results
146 indicate that the tandem fusions inside *L. monocytogenes* are intact.

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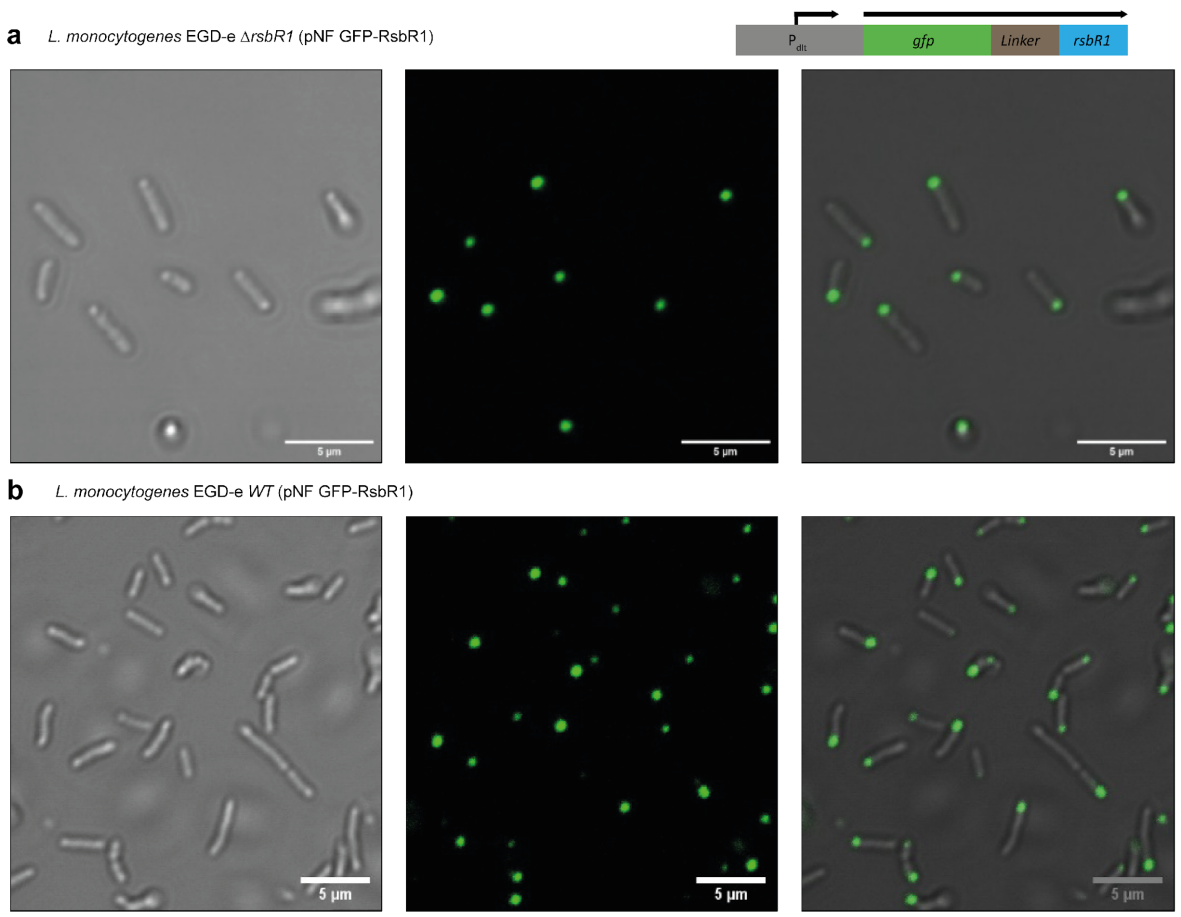
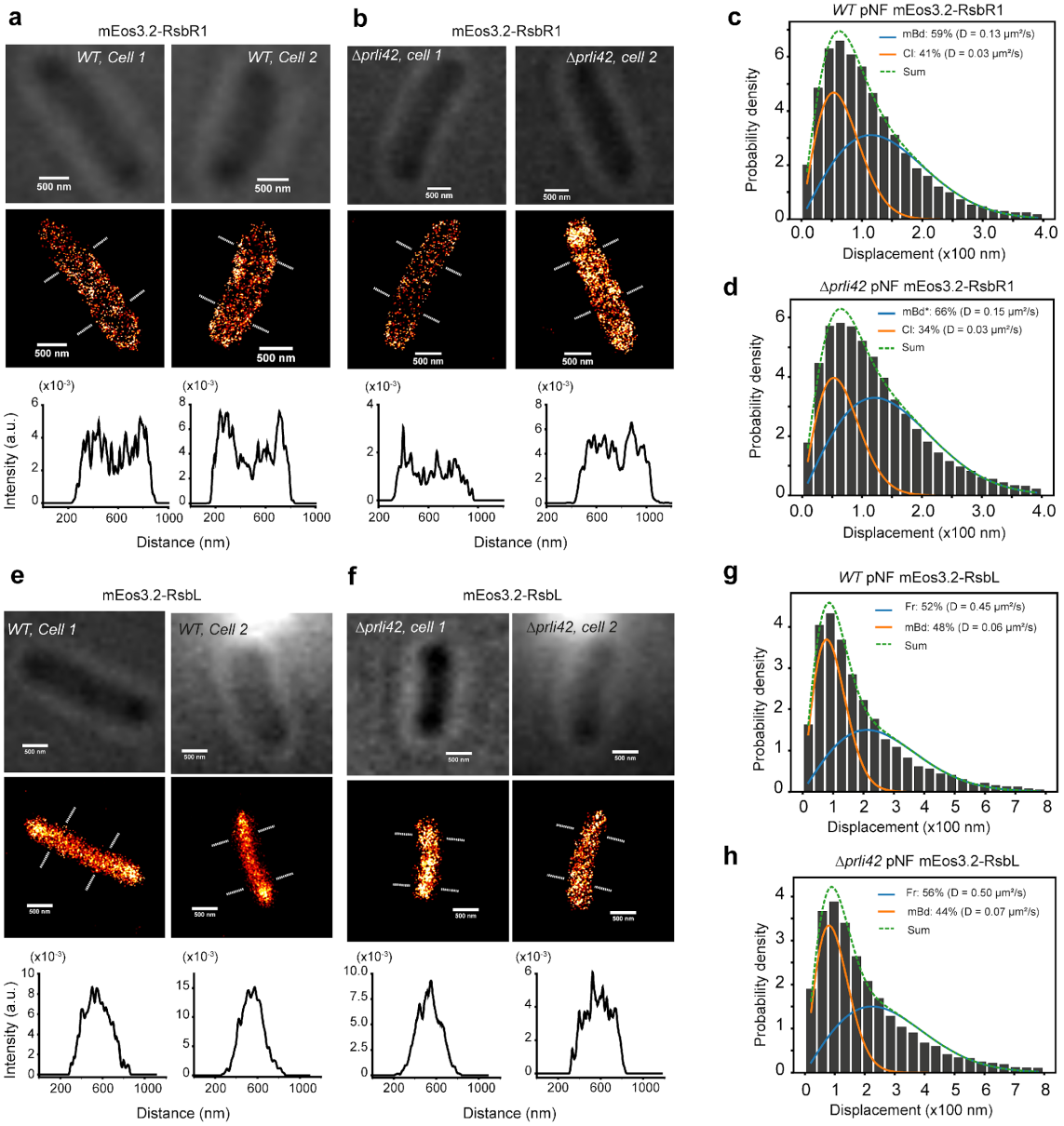


Supplementary Fig. 9 | Pole aggregation of GFP-RsbR1 observed by confocal microscopy. The green fluorescent protein GFP was fused to the N-terminal end of RsbR1 on pNF GFP-RsbR1 under the control of the P_{dlt} promoter. **a**, and **b**, The expression of *gfp-rsbR1* in *ΔrsbR1* and wild-type strains is shown, respectively. Left panels are the bright field; middle panels are the fluorescence upon 488 nm excitation; right panels are the overlays of the left and middle panels. Scale bars are 5 µm.



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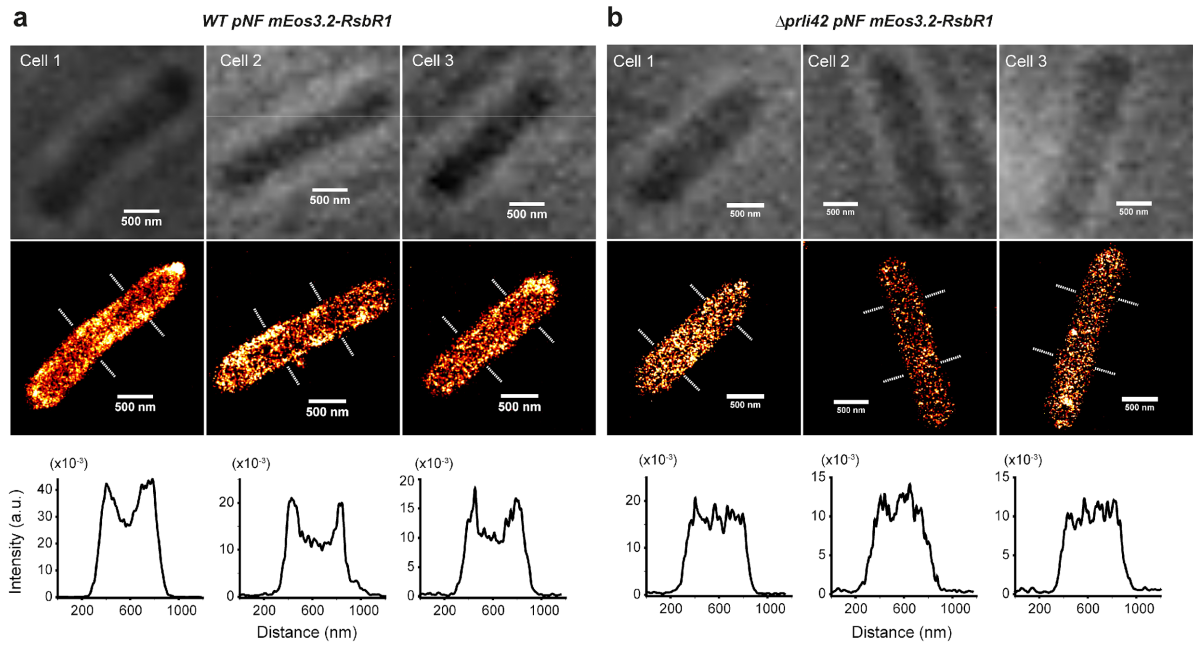
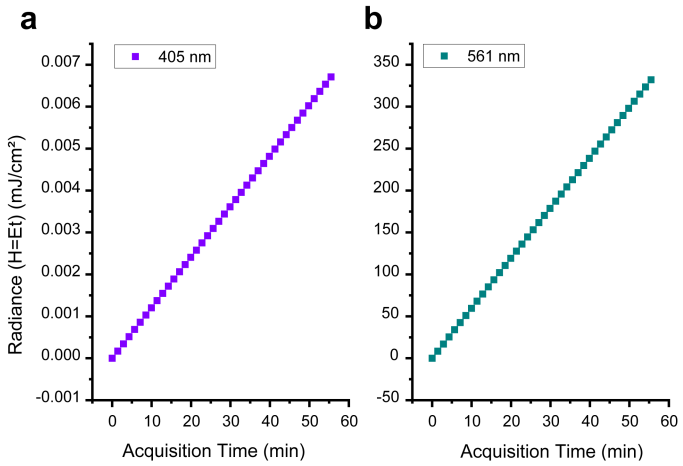
156 **Supplementary Fig. 10 | Localization by PALM and lateral diffusion by SPT of mEos3.2-**
157 **RsbR1 and mEos3.2-RsbL**. **a**, and **b**, Localization of mEos3.2-RsbR1 in wild-type and *Δprli42*
158 strains, respectively. The top panels are bright-field images. Middle panels are
159 reconstructed images from a series of frames. All fusion proteins were expressed from the
160 P_{dlt} promoter of pNF mEos3.2-RsbR1, and pNF mEos3.2-RsbL Scale bars are 500 nm. **c**, and
161 **d**, Distributions of single-molecule displacements of mEos3.2-RsbR1 in wild-type and *Δprli42*
162 strains, respectively. Blue and orange curves, showing membrane-bound (mBd) and (Cl)
163 fractions, were obtained from fitting the histogram to the two-component 2D random walk
164 model using equation (6). The starred membrane-bound fraction (mBd*) indicates mEos3.2-
165 RsbR1 molecules loosely associated with the membrane in the *Δprli42* strain. The green

dashed line is the sum of two fractions. **e**, and **f**, Localization of mEos3.2-RsbL in wild-type and *Δprli42* strains, respectively. Similar to **(c)** and **(d)**, **g**, and **h**, are distributions of single-molecule displacements of mEos3.2-RsbL. Differences in maximum displacement distances (r_{max}) were chosen based on the diffusion constants of the proteins.



Supplementary Fig. 11 | Reconstruction images of mEos3.2-RsbR1. The tandem fusion was synthesized in cells from the P_{dlt} promoter of pNF mEos3.2-RsbR1 and performing SMdM measurements (65,000 frames). Single-molecule detection was done using equation (5). **a**, Membrane localizations are detected in the wild-type strain. **b**, Most localized molecules are found in the cytoplasm in the *Δprli42* strain.



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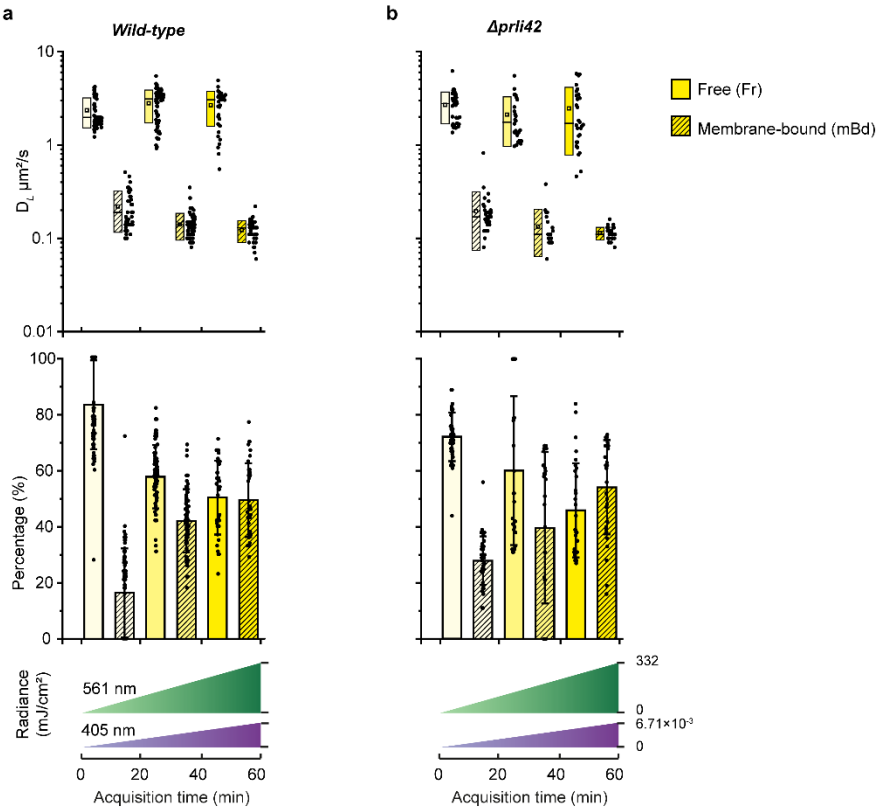
178 **Supplementary Fig. 12 | Radiance graph (H = E·t).** H is the radiant exposure dose in mJ.cm^{-}
179 ^{2}, E is the irradiance in mW.cm$^{-2,}$ and t is the exposure time in seconds. The acquisition
180 frequency is 56 Hz; the pulse time of **(a)** 405 nm and **(b)** 561 nm are 1 ms and 0.5 ms,
181 respectively; 405 nm pulses in every 2 frames, and 561 nm pulses in every frame; hence, in
182 1 second, the radiance time of 405 nm and 561 ms are 28 ms.

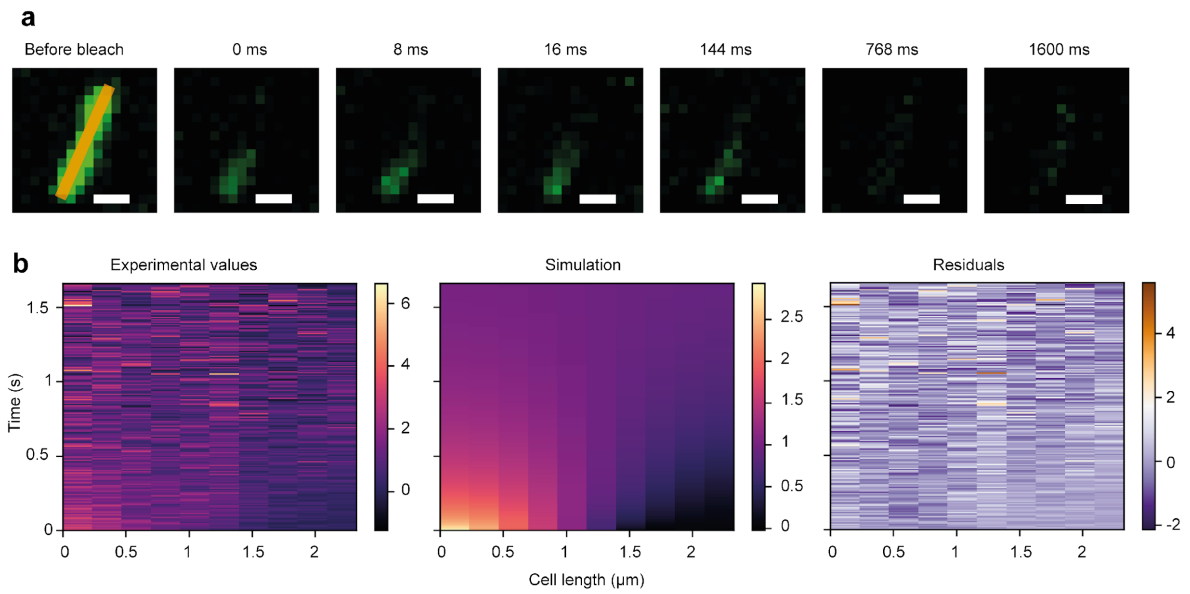
183



184

185 **Supplementary Fig. 13 | RsbL clustering upon laser light irradiation is not Prli42-**
186 **dependent. a**, and **b**, Lateral diffusion of mEos3.2-RsbL in 3 continuous SMdM acquisitions
187 (each of ~20 min) in wild-type and *Δprli42* strains, respectively. Top panel: box charts of the
188 D_L with the free (Fr) fraction and membrane-bound (mBd) fractions. Each dot shows the
189 data of one cell. Box range indicates the standard deviation (SD), and open square and dash
190 symbols inside the boxes indicate the mean and median, respectively. Middle panel: the
191 corresponding percentage of the fractions is in the top panel. Error bars represent standard
192 deviations. Bottom panel: the radiance of 561 nm and 405 nm lasers were measured at the
193 focus above the glass slide during the acquisition.

194



Supplementary Fig. 14 | FRAP acquisition to determine ensemble diffusion coefficients. **a**, An example of a FRAP acquisition and the fluorescence recovery for the diffusion constant of 1.03±0.06 $\mu m^2.s^{-1}$. The orange line marks the analyzed region; each acquisition has 200 cycles, and the average intensity before bleaching (the first 3 cycles) is used to normalize the fluorescence. Time zero was recorded immediately after the photobleaching. The scale bar is 1 μm. **b**, The fluorescent intensity along the orange line of the labelled cell of the panel (**a)** in time for the experimental data (left), the one-dimensional heat-equation simulation (middle), and the residuals (right).

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