nature portfolio

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Reporting Summary

Nature Portfolio wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Portfolio policies, see our Editorial Policies and the Editorial Policy Checklist.

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a	Confirmed
☒	☐ The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
☐	☒ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒ The statistical test(s) used AND whether they are one- or two-sided *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
☒	☐ A description of all covariates tested
☒	☐ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐	☒ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☐	☒ For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted *Give P values as exact values whenever suitable.*
☒	☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☒	☐ Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	Brightfield and fluorescence images were acquired using a microscope controlled by Hamamatsu HCImage live and LabVIEW. Details are provided in the Methods.
Data analysis	Imaging analysis was performed in MATLAB using MicrobeTracker, u-track, custom-written codes (described previously in Thrall, et al. Nat. Commun. 2017, 8, 2170). FIJI was used for visual inspection of fluorescence movies and brightfield images, but not for quantitative image analysis. Details are provided in the Methods.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our policy

The data sets from the current study are available from the corresponding author on reasonable request.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	Sample sizes for all datasets are listed in the figure captions, the associated Methods section, or in Supplementary tables. Generally, samples sizes were chosen to reliably reproduce experimental observations within reasonable margins of error. Imaging datasets represent at least 1,000 trajectories, typically collected across a few 100s of cells. Imaging replicate sizes were chosen to be sufficiently large such that measured quantities (Pol IV-SSB distance, radial distribution functions, etc) were reproducible from replicate to replicate. This produced the stated total sample sizes. We are not aware of any statistical method appropriate to determine sample size in imaging experiments, but these sample sizes are consistent with previous studies from our lab (Thrall, et al., Nat. Commun., 2017, 8, 2170) and others (Soubry, et al., PNAS, 2019, 116, 11747; Moolmann, et al., Nat Commun, 2014, 5, 5820; Hernández-Tamayo, et al., mSphere, 2021, 6, e00948-20). For in vivo TLS assays, total 300 to 1500 colonies were counted for each strain and condition in triplicate measurements. These triplicate measurements were repeated at least 3 times.
Data exclusions	For fluorescence polarization-based binding assays, weak binders that did not result in significant changes in signal at the highest concentrations (>10 uM) tested in the study were excluded for determination of binding affinities. For image analysis, individual cells were in rare cases excluded from analysis. In most cases this was done using a predetermined exclusion criterion described in the manuscript: 1. A small number of cells containing PAmCherry localizations in the first PALM frame were excluded from analysis as a precaution against cross-talk from the mYPet channel. 2. To remove a small number of poorly-fit SSB-mYPet foci, detected spots with a background level below the camera offset level (1,500 counts) were rejected. In the rare case that there was disagreement between one of the three replicates performed for each experiment (see below), an additional replicate was performed. If three replicates were in agreement, the outlier was excluded from analysis. Details are provided in the Methods.
Replication	All data have been successfully reproduced. The number of replicates and associated error are noted in the Methods section, figures, figure captions, main text, and/or Supplementary information.
Randomization	Randomization was not relevant to this study.
Blinding	Blinding was not relevant to this study.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☐	☒ Animals and other organisms
☒	☐ Human research participants
☒	☐ Clinical data
☒	☐ Dual use research of concern

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☐	☒ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used	1) anti-LacI (antibodies-online Inc. ABIN964896), 2) anti-FLAG (a gift from the Walter lab in Harvard medical school), 3) anti-RpoA (BioLegend, #663104, clone 4RA2), 4) anti-rabbit IgG-HRP (Jackson ImmunoResearch, #111-035-003), 5) anti-mouse IgG-HRP (Jackson ImmunoResearch, #315-035-003). The dilutions of antibodies used for this study are specified in the Methods section.
Validation	All but the anti-FLAG antibody are commercial and were validated by the manufacturers and other researchers. The anti-LacI and anti-RpoA antibodies were validated for Western blotting with purified recombinant E. coli LacI and E. coli BL21 cell lysates

respectively by the manufacturers. Anti-FLAG antibody was raised against the Ac-C(dPEG4)DYKDDDDK-OH and validated by the Walter group in Harvard medical school (Ref. Nature. 567, 267-272, 2019).

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals	E. coli (MG1655)
Wild animals	This is not relevant to this study.
Field-collected samples	This is not relevant to this study.
Ethics oversight	All the experiments using E. coli (MG1655) were performed in a BL1 laboratory by following the relevant biosafety protocol .

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Flow Cytometry

Plots

Confirm that:

☒ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).

☒ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).

☐ All plots are contour plots with outliers or pseudocolor plots.

☐ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation	Formaldehyde-fixed E. coli cells were washed multiple times with PBS and resuspended in PBS for flow cytometry
Instrument	Accuri C6 (BD Biosciences)
Software	BD Accuri C6 software (built-in software for Accuri C6)
Cell population abundance	No sorting was performed. More than 90 X 10^3 cells were included for each strain.
Gating strategy	No gating.

☐ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.