Supporting information for

Real-Time Nanoscale Investigation of Spore Coat Assembly in *Bacillus subtilis*

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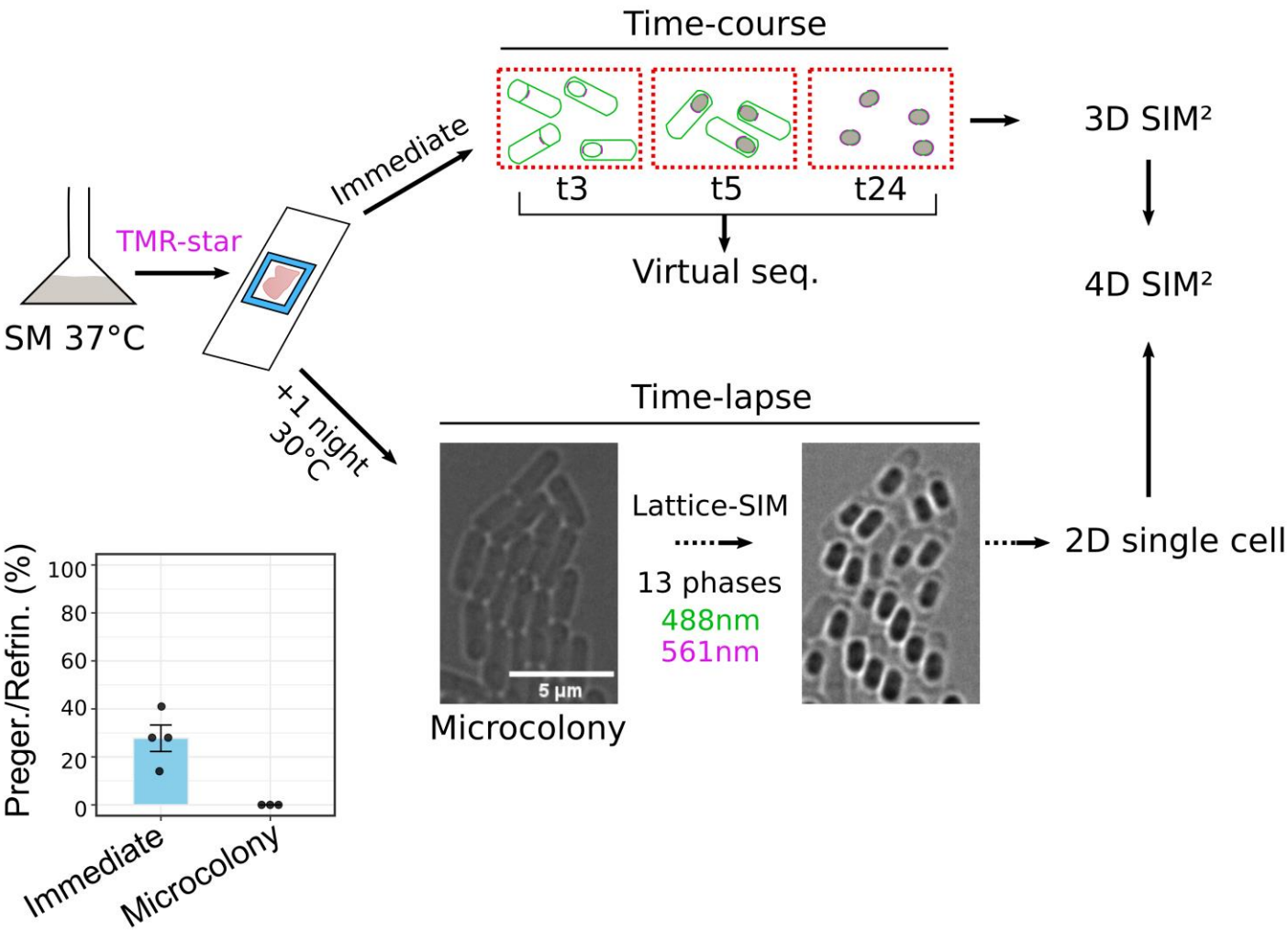
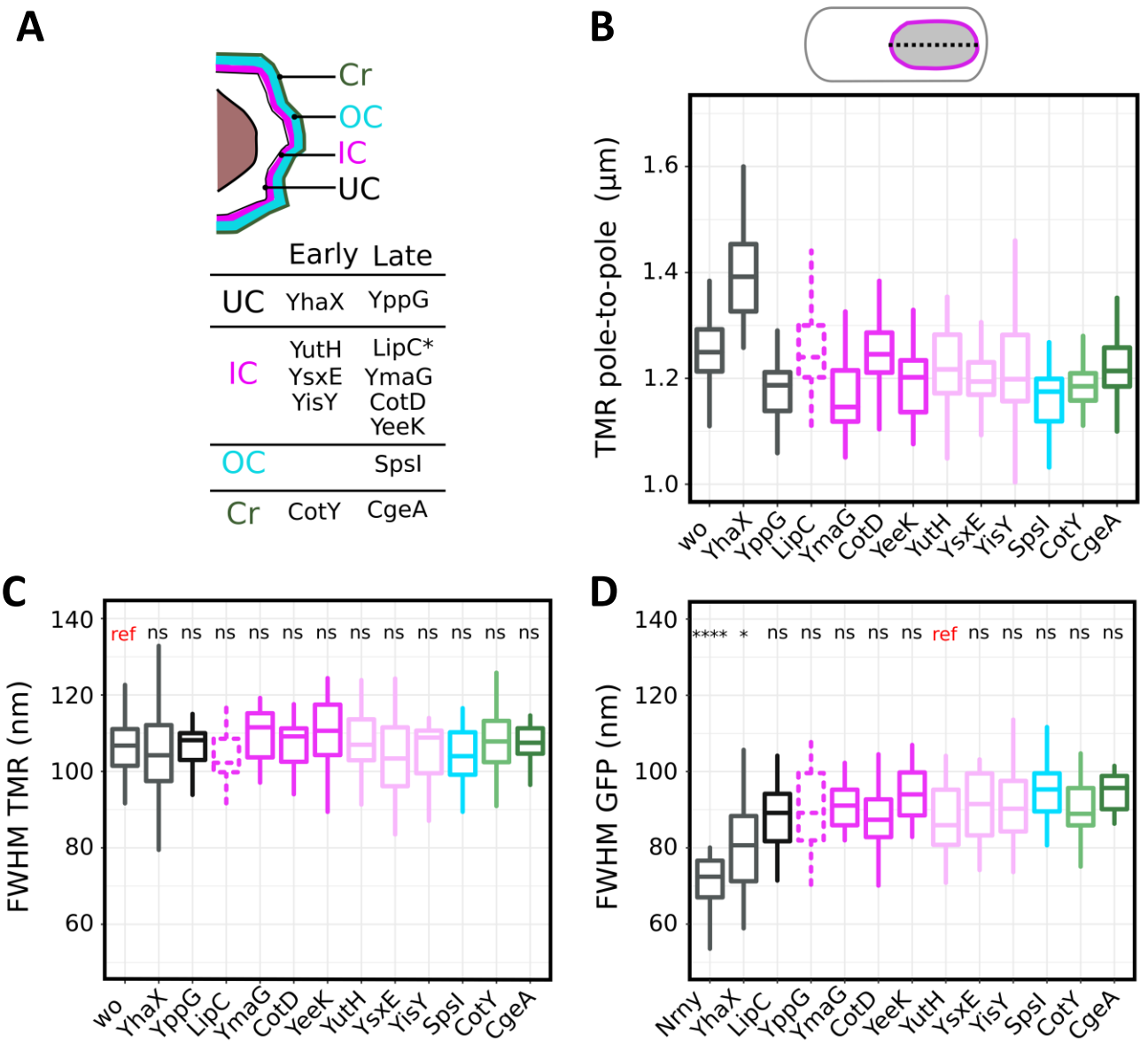
Supplementary Table S1 Strains used in this study

Strains	Genotype	Source
PY79	Prototrophic derivative of *B. subtilis* 168	(Youngman et al., 1984)
PE479	yutHΩpPE61 (yutH-gfp spc)	(van Ooij et al., 2004)
PE3158	amyE::P_{cotYZ}cotY-gfp cat	(Shuster et al., 2018)
PE2708	spsIΩspsI-gfp spc	(Arrieta ortiz et al., 2015)
PE789	yeeKΩpKW33 (yeeK-gfp spc)	(Wang et al., 2009)
PE659	cotDΩpKW8 (cotD-gfp spc)	(Wang et al., 2009)
CF55	ymaGΩpCF43 (ymaG-gfp spc)	(Kim et al., 2006)
PE388	yhaXΩpPE42 (yhaX-gfp spc)	(Eichenberger et al., 2003)
CCB861	W168 ΔsigK::tet kapD:GFP-pCV0119	(D'Halluin et al., 2025)
BKK28410	ΔgerE::kan	(Koo et al., 2017)
BKK15170	ΔspoVD::kan	(Koo et al., 2017)
CCB911	ΔspoIVA::ery kapD:GFP-pCV0119	(D'Halluin et al., 2025)
CCB909	ΔsafA::ery kapD:GFP-pCV0119	(D'Halluin et al., 2025)
CCB908	ΔcotE::ery kapD:GFP-pCV0119	(D'Halluin et al., 2025)
CCB905	ΔcotZ::ery kapD:GFP-pCV0119	(D'Halluin et al., 2025)
PE485	yisYΩpPE66 (yisY-gfp spc)	(Kim et al., 2006)
CF54	yxeEΩpCF45 (yxeE-gfp spc)	(Kim et al., 2006)
PE1146	lipCΩpKW40 (lipC-gfp spc)	(Wang et al., 2009)
PE491	yppGΩpPE82 (yppG-gfp spc)	(Eichenberger et al., 2003)
PE601	ysxEΩpPG1 (ysxE-gfp spc)	(Kim et al., 2006)
PE3260	amyE::P_{cgeAB}cgeA-gfp cat	(Shuster et al., 2018)
RCL1720	ΔsigK::tet, yutH-gfp spc	CCB861 > PE479
RCL1696	ΔgerE::kan, yutH-gfp spc	BKK28410 > PE479
RCL1697	ΔspoVD::kan, yutH-gfp spc	BKK15170 > PE479
RCL1787	ΔspoIVA::ery, yutH-gfp spc	CCB911 > PE479
RCL1698	ΔsafA::ery, yutH-gfp spc	CCB909 > PE479
RCL1699	ΔcotE::ery, yutH-gfp spc	CCB908 > PE479
RCL1700	ΔcotZ::ery, yutH-gfp spc	CCB905 > PE479
RCL1736	amyE::P_{rny*}Nrny-sfGFP spc	pDG1730-RnysfGFP > PY79



Supplementary Figure S1. Workflow of lattice-SIM² imaging of *Bacillus subtilis* sporulation. (A) Schematic of the experimental procedures used for time-course (top panels) or time-lapse (bottom panels) analysis of *B. subtilis* sporulation. For time course experiments, cells resuspended in sporulation medium (SM) are harvested at different time points during the time-course of sporulation. Previously, a virtual sequence of coat proteins assembly was derived from fluorescence images of sporangia population. Here, we labeled the cells with TMR-star, and imaged immediately with 3D lattice-SIM². For time-lapse at the single-sporangium level, we performed lattice-SIM microscopy of cells sporulating in microcolonies. Cells were collected 5.5 hours after resuspension, labelled with TMR-star, mounted on an agarose-coated microscope slide and incubated overnight at 30° C. Non-sporulating cells multiply, forming microcolonies on the agarose pad. Sporulation occurring in those microcolonies is highly efficient, as indicated by development of refractile forespores, despite repeated and intense laser exposure. Up to four strains can be imaged in parallel for up to 12 hours. Following SIM² reconstruction and post-processing corrections of Lattice SIM acquisitions (see Methods), quantitative parameters such as protein encasement speed can be extracted. Furthermore, by monitoring the single-sporangium 2D sequences of GFP-tagged coat protein assembly (488 nm laser) relative to the TMR-star signal (561 nm laser), a super-resolved 4D assembly sequence can be proposed. **(B)** A negligible fraction of sporangia exhibited premature germination event in cells sporulating in microcolonies compared to cells sporulating in liquid medium and collected for immediate visualization. The graph shows the percentage of premature germination events ("Preger.") among the population of refractile sporangia ("Refrin."). Experiments were performed at least 3 times for the two conditions. See also Supplementary movie S1.



Supplementary Figure S2. Effect of expression of GFP fusions to coat proteins on spore dimensions and TMR-star signal. (A) Schematic indicating the timing of synthesis (early or late) and the localization of various GFP fusions to indicated coat proteins (wo, PY79; YhaX, PE388; YppG, PE491; LipC, PE1146; YmaG, CF55; CotD, PE659; YeeK, PE789; YutH, PE479; YsxE, PE601; YisY, PE485; SpsI, PE2708; CotY, PE3158; CgeA, PE3260), according to Driks and Eichenberger (2016). *, Note that LipC localization was unclear in this previous study (magenta dotted boxplots in **B-D**), being classified as a basement layer protein (Driks and Eichenberger, 2016). Here, the extensive colocalization of LipC-GFP with TMR-star, similar to other late-synthesized inner coat proteins and in contrast to the basement layer YppG reporter (**see Supplementary Fig. S5**), supports that LipC is an inner coat protein. **(B)** Quantification of the sporangium length (from MCP pole to MCD pole) based on the TMR-star signal in sporangia expressing the indicated GFP-fusions. At least 22 sporangia per condition were analyzed, except for the YppG condition (n=12). A schematic of a sporangium with a dotted line indicating the region used for quantification is shown at the top. wo, without GFP-fusion expressed. **(C)** Full Width at Half Maximum (FWHM) quantification of TMR-star signal in refractile sporangia expressing the indicated GFP-fusions. At least 12 sporangia were analyzed per condition. The non-parametric, two-sided, Mann–Whitney U test was used with "wo" as a reference (ref); ns: not significant, $P > 0.05$. **(D)** FWHM quantification of GFP-coat fusions signal in refractile sporangia expressing the indicated GFP-fusions. At least 12 sporangia were analyzed per condition. The non-parametric, two-sided, Mann–Whitney U test was used with strain expressing YutH-GFP as a reference. ns: not significant, $P > 0.05$; *, $P<0.05$; ****, $P<0.00005$.

A

BamHI-spacer-PymdA-**Nrny[1-30]**-sfGFP-spacer-EcoRI

GGATCCGATCCTGTATTACTATTCTTAGTTAAGATGGCAAGCTTGACAAGTATTTCCGACACATTTACA
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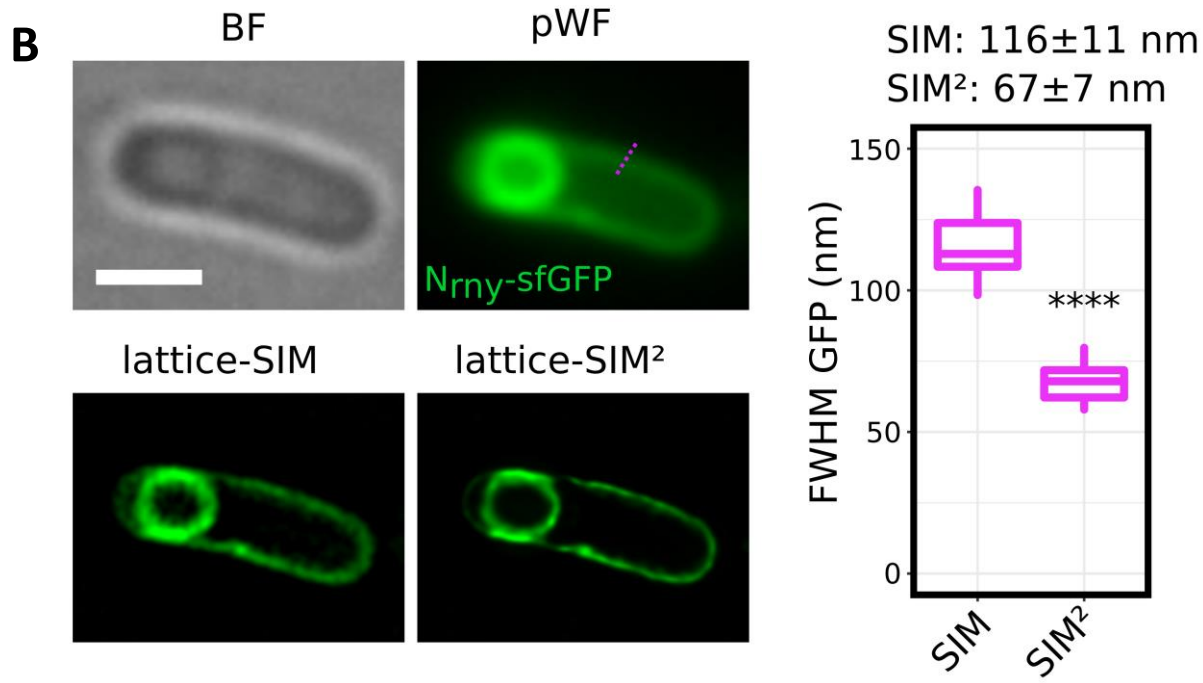
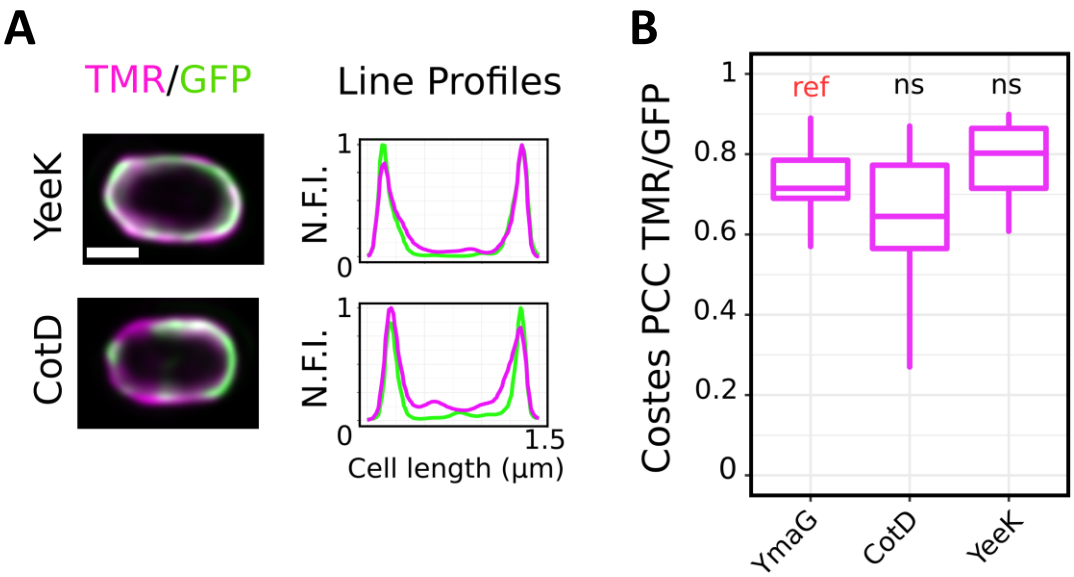
TC

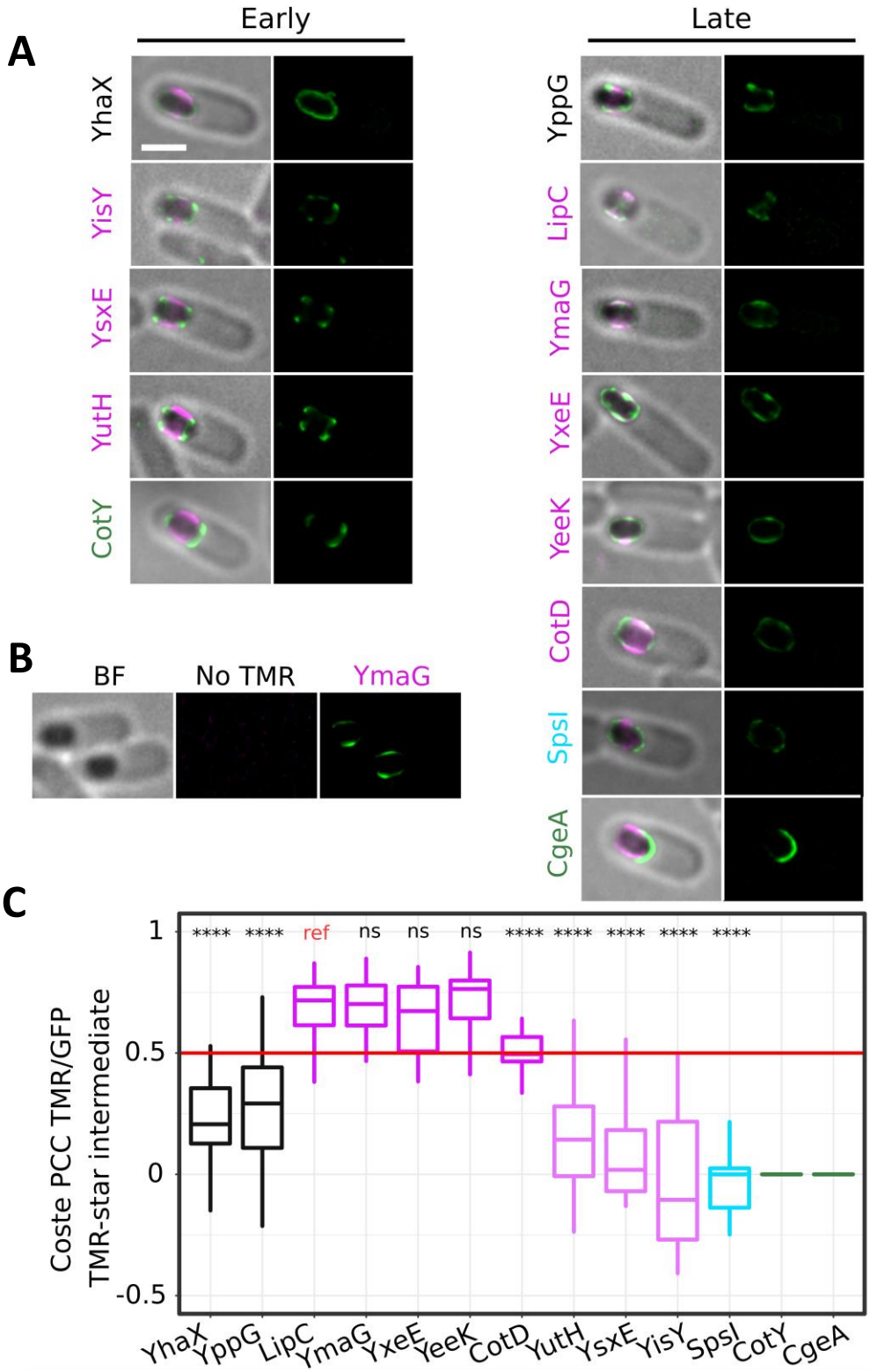
B



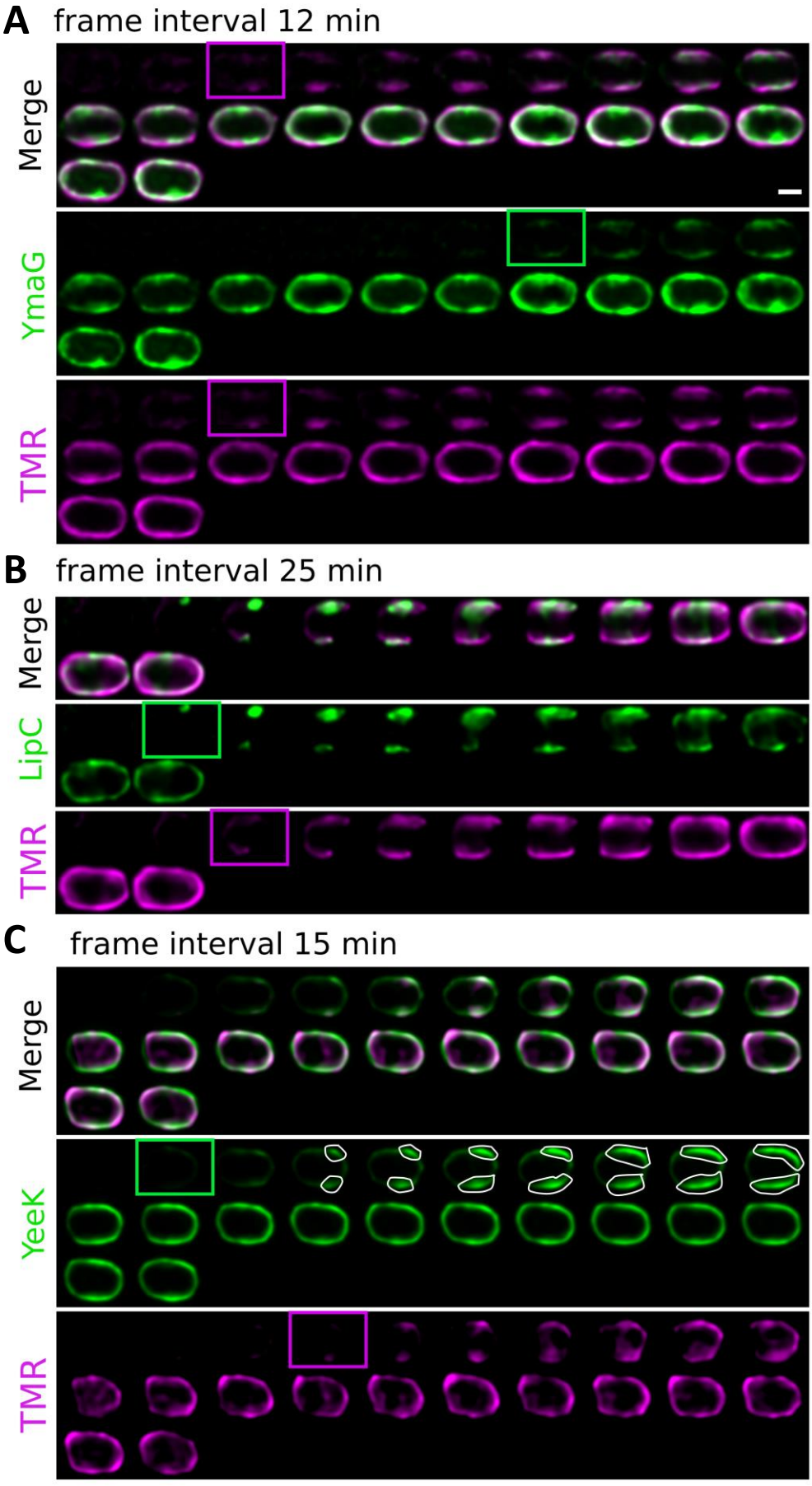
Supplementary Figure S3. Assessment of lattice-SIM² lateral resolution. (A) DNA sequence of the Rny single pass N-terminal helix domain (amino acid 1-30) fused to sfGFP cloned into pDG1730 plasmid using BamHI/EcoRI sites and integrated at the *amyE* locus by a double recombination event (RCL1736). A synthetic promoter derived from the natural PymdA promoter was used (Guiziou *et al*., 2016). **(B)** Comparison of pseudo-widefield (pWF), lattice-SIM and lattice-SIM² image of strain RCL1736 expressing the Nrny-sfGFP fusion as a membrane reporter, collected 5.5 hours after resuspension in sporulation medium. Brighfield (BF) illumination allows to distinguish engulfing membranes. The magenta dotted line in the pWF panel indicates the region used for quantification in panel C. **(C)** Mean and standard deviation of the quantification of FWHM as a proxy for lateral resolution of Nrny-sfGFP membrane signal (n=15). The non-parametric, two-sided, Mann–Whitney U test was used with the "SIM" condition as a reference; ****, P<0.00005. Scale bar is 1µm.



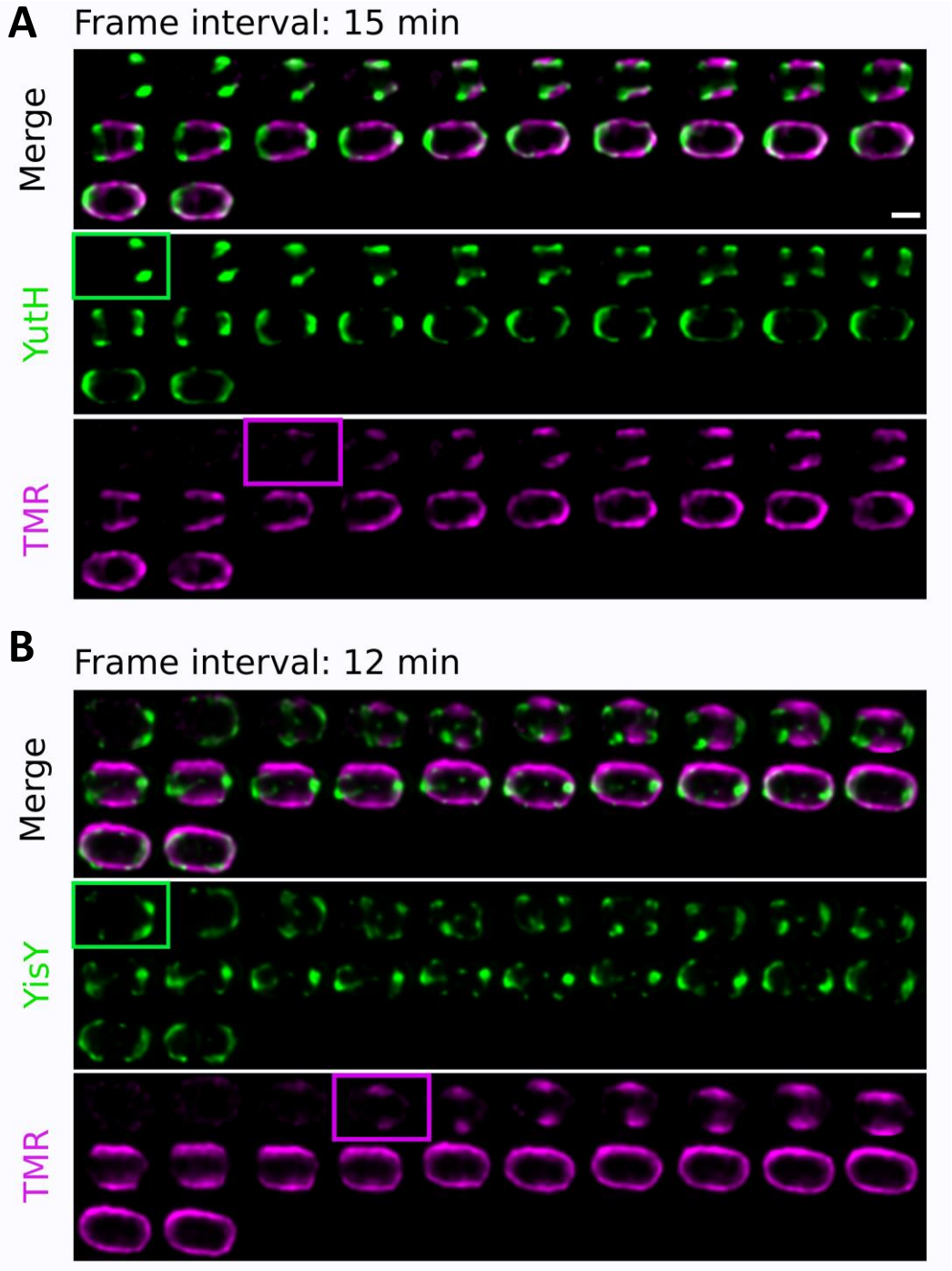
Supplementary Figure S4. TMR-star colocalizes with the late-synthesized inner coat proteins YeeK and CotD. (A) Representative micrographs showing co-localization of the late-synthesized inner coat proteins YeeK (strain PE789) and CotD-GFP (strain PE659) relative to TMR-star when it fully covers the forespore surface. Scale bar, 500 nm. Line profiles analysis (from pole to pole) show the superposition of GFP and TMR-star normalized signals. N.F.I, Normalized Fluorescence Intensity. **(B)** Distribution of Costes Pearson Correlation Coefficient (Costes PCC) between signals of TMR-star and GFP fused to the indicated coat proteins. The non-parametric, two-sided, Mann–Whitney U test was used with YmaG-GFP as a reference (ref); ns: not significant, $P > 0.05$ (at least 22 sporangia per condition).



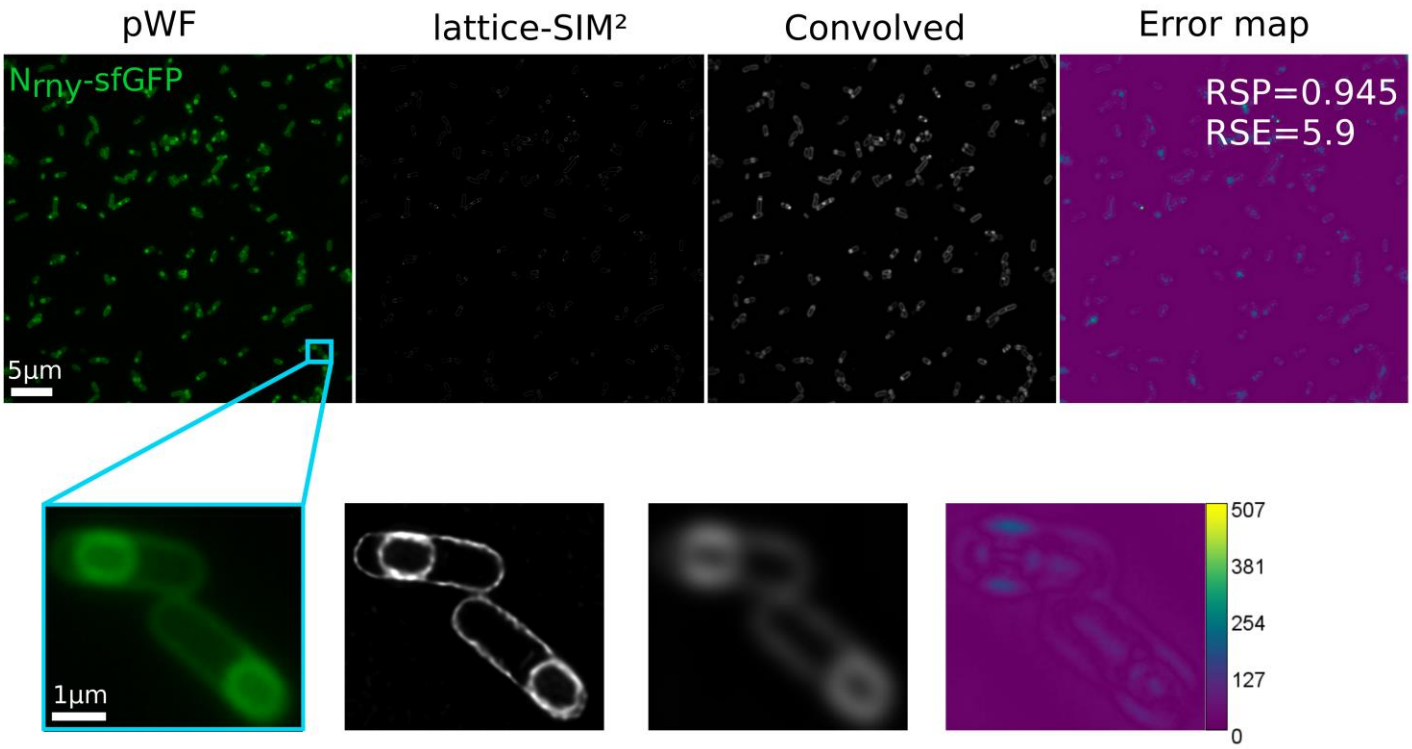
Supplementary Figure S5. Analysis of various coat proteins localization at an intermediate stage of TMR-star assembly. (A, B) Representative super-resolution micrographs showing localization of the indicated early-synthesized or late-synthesized coat protein fused to GFP (YhaX, PE388; YppG, PE491; LipC, PE1146; YmaG, CF55; CotD, PE659; YeeK, PE789; YxeE, CF54; YutH, PE479; YsxE, PE601; YisY, PE485; SpsI, PE2708; CotY, PE3158; CgeA, PE3260), when TMR-star is only detected on the midspore region and not detected towards the forespore poles. (A) and control of YmaG-GFP localization in absence of TMR-star labelling **(B)**. Sporangia were collected between hours 5.5 and 7 after resuspension in sporulation medium. Scale bar, 500 nm; BF, bright-field. **(C)** Distribution of Costes Pearson Correlation Coefficient (Costes PCC) between signals of TMR-star (only viewed in midspore region) and GFP fused to the indicated coat proteins over the entire FS surface. For the crust proteins CotY and CgeA, the software was not able to compute a Costes PCC because of a complete absence of correlation between GFP and TMR-star signals in these conditions. PCC=1 would indicate perfect colocalization; PCC=0, no colocalization; PCC>0.5 is considered as a significant colocalization (ref line). The non-parametric, two-sided, Mann–Whitney U test was used with LipC-GFP as a reference (ref); ns: not significant, $P > 0.05$; ****, $P<0.00005$ (at least 16 sporangia per condition).



Supplementary Figure S6. Single-sporangium dual color Lattice-SIM² time-lapse showing the localization of the late-synthesized inner coat proteins YmaG, LipC and YeeK relative to the TMR-star signal. Original time intervals of the time-lapse acquisition are indicated. Green and magenta squares indicate the first frame of GFP-coat fusion (LipC, PE1146; YmaG, CF55; YeeK, PE789) and TMR-star detection, respectively. For YeeK-GFP, the area of increased intensity that followed the assembly of TMR-star is highlighted with a white outline. Scale bar, 500 nm.



Supplementary Figure S7. Single-sporangium dual color Lattice-SIM² time-lapse showing the localization of the early-synthesized inner coat proteins YutH and YisY relative to the TMR-star signal. Original time intervals are indicated. Green and Magenta squares indicate the first frame of GFP-coat fusion (YutH, PE479; YisY, PE485) and TMR-star detection respectively. Scale bar, 500 nm.



Supplementary Figure S8. Control of Lattice-SIM² reconstructions using SQUIRREL analysis. Sporulating cells expressing Nrny-sfGFP fusion (RCL1736) collected 5.5 hours after resuspension were imaged using lattice-SIM and reconstructed with the SIM² algorithm as described in the Methods section. A pseudo-widefield (pWF) image used as a 'Raw' image, a lattice-SIM² super-resolution reconstruction, a super-resolution image convolved with an automatically computed RSF ('Convolved'), and a quantitative map of errors between reference and convolved SR images ('Error map'; color bar indicates magnitude of the error) are depicted. The error maps show the resolution-scaled error (RSE), calculated between the blurred super-resolution lattice-SIM² image and the pWF image. The resolution-scaled Pearson coefficient (RSP) represents a normalized quality metric, showing high values and thus high-quality reconstructions for lattice-SIM² reconstructions. An inset showing two sporulating cells is shown in the bottom panels.