Figures and figure supplements

General principles for the formation and proliferation of a wall-free (L-form) state in bacteria

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Figure 1. Inhibition of PG precursor synthesis induces L-form proliferation in bacteria. (A) Schematic model of peptidoglycan (PG) precursor (lipid II) synthesis in bacteria and its inhibition by the antibiotics fosfomycin (FOS) and D-cycloserine (DCS). MurA, inhibited by the antibiotic FOS, and MurB catalyze the transformation of uridine diphosphate-N-acetylglucosamine (UDP-GlcNAc) into UDP-N-acetylmuramic acid (UDP-MurNAc). The racemase Dal and the D-alanine ligase Ddl, both of which are inhibited by the antibiotic DCS, are required to generate D-Ala-D-Ala. This is incorporated into the UDP-MurNAc-pentapeptide, requiring MurC, MurD, MurE, and MurF enzymes. UDP-MurNAc-pentapeptide is transferred to undecaprenyl pyrophosphate by MraY, and the addition of GlcNAc is catalyzed by MurG to form lipid II. (B) Growth of Bacillus subtilis strain LR2 (ispA\_P\_xyl\_murE-B) streaked on L-form supporting medium (MSM) or nutrient agar (NA) plates in the presence (lipid II ON) or absence (lipid II OFF) of 0.5% xylose. (C) Phase contrast microscopy of B. subtilis LR2 cells grown on MSM plates in the presence (left) or absence (right) of 0.5% xylose. (D–I) Growth on plates (D, F, H) and corresponding phase contrast microscopy (E, G, I) of bacterial strains Staphylococcus aureus ATCC2913 (D, E), Corynebacterium glutamicum ATCC13032 (F, G), and Escherichia coli MG1655 (H, I). (D, F, H) The different bacterial strains were streaked on MSM or NA plates in the absence (lipid II ON) or presence (lipid II OFF) of the antibiotics FOS (D, H) or DCS (F). (E, G, I) Phase contrast microscopy of the different bacterial cells grown on MSM plates in the absence (left) or presence (right) of the antibiotics FOS (E, I) or DCS (G). Scale bars, 3 μm. DOI: 10.7554/eLife.04629.003
Figure 1—figure supplement 1.
*Bacillus subtilis* L-form growth requires an additional mutation in a gene such as *ispA*. Growth of *B. subtilis* strain BS115 (*P*<sub>xyl</sub>-murE-<i>B</i>) streaked on L-form supporting medium (MSM) in the absence (lipid II OFF) of 0.5% xylose.
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Figure 1—figure supplement 2. Bacterial L-forms proliferate on β-lactams. (A) Growth of Staphylococcus aureus (left), Corynebacterium glutamicum (middle), and Escherichia coli (right) walled strains streaked on L-form supporting medium (MSM) in the presence of penicillin G (S. aureus and C. glutamicum) or ampicillin (E. coli). (B) Growth of S. aureus (top), C. glutamicum (middle), and E. coli (bottom) L-forms streaked on MSM with the antibiotics fosfomycin (S. aureus and E. coli) or D-cycloserine (C. glutamicum) in the presence of penicillin G (S. aureus and C. glutamicum) or ampicillin (E. coli). The different L-form strains were streaked several times under the same conditions every 3 days (left to right).
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Figure 1—figure supplement 3. Bacterial L-form cell wall reversion. (A) Cell wall reversion on L-form supporting medium (MSM) plates of L-forms of *Escherichia coli* strain TB28 grown on MSM plates containing fosfomycin and ampicillin. (B) Growth of the *E. coli* L-form reverted strain TB28 from panel (A) on nutrient agar (NA). (C) Cell wall reversion on MSM plates of L-forms of *Corynebacterium glutamicum* grown on MSM plates with D-cycloserine and penicillin G. (D) Growth of the *C. glutamicum* L-form reverted strain from panel (C) on NA.

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Figure 2. *E. coli* L-forms proliferate independently of the peptidoglycan cell wall machinery. (A) Growth of the *Escherichia coli* strains TB28 (top) and RM345 (ΔmurA, bottom) containing the unstable plasmid pOU82-Amp-murA streaked on nutrient agar plates in the presence of X-gal. (B) L-form colonies of the *E. coli* strains RM345 (ΔmurA, pOU82-Amp-murA) on L-form supporting medium (MSM) plates in the presence of fosfomycin (FOS) and X-gal, after several repeated streakings on MSM plates in the presence of FOS. (C) Multiplex PCR of the *ftsK, murA, ftsZ,* and *mreC* genes from genomic DNA of the *E. coli* strains RM345 grown in the walled (1) or L-form (2) states, obtained from the strains in panel (B). M represents the 100 bp DNA ladder.

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Figure 3. Mode of cell division of E. coli and C. glutamicum L-forms. (A, B) Corynebacterium glutamicum L-form strain grown in nutrient broth (NB)/L-form supporting medium (MSM) with D-cycloserine (A), and Escherichia coli L-form strain RM345 (ΔmurA) grown on nutrient agar/MSM (B), were observed by time lapse phase contrast microscopy. Elapsed time (min) is shown in each panel. Scale bars, 3 μm. Arrows represent the direction of protrusion formation and the asterisks (*) the daughter cells after division. See also Videos 1–4. DOI: 10.7554/eLife.04629.008
Figure 4. Bacterial L-forms proliferate independently of the cell division machinery. (A) Growth of the *Escherichia coli* strains TB28 (top) and RM349 (ΔftsZ, bottom) containing the unstable plasmid pOU82-Amp-ftsZ streaked on nutrient agar plates in the presence of X-gal. (B) L-form colonies of the *E. coli* strains RM349 (ΔftsZ, pOU82-Amp-ftsZ, top left), RM350 (ΔmurA, ΔftsZ, pOU82-Amp-ftsZ, pSK122-Cm-murA, top right), RM61 (ΔftsK, pSK122-Cm-ftsK, bottom left), and RM359 (ΔmreBCD, pHMB2-Kn-mreBCD) on L-form-supporting medium (MSM) plates in the presence of fosfomycin (FOS) and X-gal, after several repeated streakings on MSM plates in the presence of FOS. (C) Multiplex PCR of the ftsK, murA, ftsZ, and mreC genes from genomic DNA of the *E. coli* strains RM349 (1, 2), RM350 (3, 4), RM61 (5, 6), and RM359 (7, 8) grown in the walled (1, 3, 5 and 7) or L-form (2, 4, 6 and 8) states obtained from the strains in panel (B). M represents the 100 bp DNA ladder. (D) Growth of the *Staphylococcus aureus* strain RNApFtsZ-1 (erm-pSPAC-ftsZ, *Pinho and Errington, 2003*) streaked on MSM plates in the absence (lipid II ON, left) or presence (lipid II OFF, middle and right) of FOS, with (+FtsZ, middle) or without (−FtsZ, left and right) isopropyl β-D-thiogalactopyranoside. (E) Growth profiles of *Corynebacterium glutamicum* in MSM with (L-form state, right, lipid II OFF) or without (walled Figure 4. Continued on next page
state; left, lipid II ON) D-cycloserine, and in the absence
(red) or presence (blue) of cephalexin.
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Figure 4. Continued

Figure 4—figure supplement 1. Staphylococcus
aureus L-forms proliferate in the absence of the cell
division machinery. (A) Cell wall reversion on L-form
supporting medium (MSM) plates with isopropyl
β-D-1-thiogalactopyranoside (IPTG) (+FtsZ) of L-forms
of S. aureus strain RNpFtsZ-1 grown on MSM plates
with fosfomycin (FOS) and without IPTG, obtained from
Figure 4D, right. (B) Growth of the S. aureus RNpFtsZ-1
L-form reverted strain from panel (D) on nutrient agar
plates with (+FtsZ) or without (−FtsZ) IPTG. (C) Growth
of the S. aureus strain ATCC2913 ftsZR191P (Haydon et
al., 2008) streaked on MSM plates with (lipid II ON, left
and middle) or without (lipid II OFF, right) FOS, and in
the presence (left) or absence (middle and right) of
benzamide.
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Figure 4—figure supplement 2. Corynebacterium
glutamicum L-forms proliferate in the absence of the
cell division machinery. Growth of the C. glutamicum
strain streaked on nutrient agar (NA) (lipid II ON) in the
absence (left, −cephalexin) or presence (middle,
+cephalexin) of cephalexin, and on L-form supporting
medium (MSM) plates with D-cycloserine and cephalex-
in (right, lipid II OFF, + cephalexin).
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Figure 5. Essential role of fatty acid synthesis in L-forms growth of *E. coli* and *C. glutamicum*. (A) Growth of *Escherichia coli* strains RM365 (∆*fabH*) and RM366 (∆*fabH*, pCA24N-*fabH*) streaked on L-form supporting medium (MSM) in the absence (lipid II ON) or presence (lipid II OFF) of fosfomycin (FOS). (B) L-form colonies of the *E. coli* strain RM369 (∆*murA*, pSK122-Cm-*ftsK*, ∆*fabH*, pOU82-Amp-*fabH*) on MSM plates after several repeated streakings on MSM plates in the presence of FOS. (C) Multiplex PCR of the genes, *murA*, *fabH*, and *mreC* on genomic DNA of the *E. coli* strain RM369 grown in the walled (1) or L-form (2) state. Samples obtained from strains in panel (B). M represents the 100 bp DNA ladder. (D) Growth of *Corynebacterium glutamicum* streaked on MSM in the absence (lipid II ON) or presence (lipid II OFF) of D-cycloserine (DCS), and with (cerulenin) or without (no) 2 μg/ml of cerulenin. (E) Typical images of *C. glutamicum* L-forms after 16 hr of growth in MSM with DCS in the absence (−cerulenin) or presence (+cerulenin) of 2 μg/ml of cerulenin. Scale bars, 3 μm. See also Videos 5 and 6. DOI: 10.7554/eLife.04629.016
Figure 5—figure supplement 1. Specific inhibition of *Escherichia coli* L-forms growth by cerulenin. Growth of *E. coli* walled (top) and L-form (bottom) strains streaked on L-form supporting medium (MSM) in the absence (lipid II ON) or presence (lipid II OFF) of fosfomycin with different concentration of cerulenin (0, 10, and 20 μg/ml).
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