ARTICLES

Life without a wall or division machine in *Bacillus subtilis*

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The cell wall is an essential structure for virtually all bacteria, forming a tough outer shell that protects the cell from damage and osmotic lysis. It is the target of our best antibiotics. L-form strains are wall-deficient derivatives of common bacteria that have been studied for decades. However, they are difficult to generate and typically require growth for many generations on osmotically protective media with antibiotics or enzymes that kill walled forms. Despite their potential importance for understanding antibiotic resistance and pathogenesis, little is known about their basic cell biology or their means of propagation. We have developed a controllable system for generating L-forms in the highly tractable model bacterium *Bacillus subtilis*. Here, using genome sequencing, we identify a single point mutation that predisposes cells to grow without a wall. We show that propagation of L-forms does not require the normal FtsZ-dependent division machine but occurs by a remarkable extrusion-resolution mechanism. This novel form of propagation provides insights into how early forms of cellular life may have proliferated.

Binary fission is a widely conserved mechanism required for the proliferation of almost all cells. In bacteria, the central protein in cytokinesis is the bacterial tubulin homologue, FtsZ (filamentous temperature sensitive protein Z), which forms a ring (the Z-ring) to which other proteins are recruited[1]. The division machine then constricts in parallel with synthesis of a cross wall that matures into the new polar caps of the daughter cells[2] (Supplementary Fig. 1a). The machine includes enzymes called penicillin-binding proteins, which synthesize peptidoglycan, the major component of the wall[3] (Supplementary Fig. 1b). L-form bacteria[4,5] can proliferate but are thought not to have any wall; they offer an interesting model for investigating the details of Z-ring function[6], particularly whether constriction of the Z-ring requires wall synthesis.

A rapid method for generating L-forms

Existing L-form bacteria are difficult to manipulate[7], and modern genetic tools such as inducible promoters and green fluorescent protein (GFP) fusions have not been used in them. We therefore developed an approach to reproducibly generate stable *B. subtilis* L-forms.

We used strain M96 in which expression of the *murE* operon, which encodes several enzymes essential for the synthesis of the precursor of peptidoglycan[8], is controlled by the xylose inducible promoter P_{xyl}. In the presence of inducer, both the growth rate and the shape of this strain were normal (Fig. 1a–c). In the absence of inducer, cell growth was arrested and cells bulged and lysed (Fig. 1a, d). As previous work has shown that many shape mutants can be rescued by high concentrations of Mg^{2+} or osmoprotectants[9–12], we tested whether these conditions could restore growth to the MurE depletion strain. In the presence of high concentrations of Mg^{2+}, the growth of this strain was restored to a limited extent (Fig. 1a). Phase contrast microscopy of cells incubated in medium containing Mg^{2+} and sucrose revealed that lysis was reduced, and large, amorphous cells accumulated (Fig. 1e) that were similar in appearance to classically derived[7,13,14] and antibiotic induced L-forms[6]. These cells appeared to have little or no wall: when squashed between a microscope slide and a cover slip they packed together in a tessellated pattern, showing not only that they had lost their shape but also the rigidity of their envelope (Fig. 1f).

Unfortunately, these cells did not undergo sustained proliferation and the cultures were rapidly overrun with rod-shaped cells (Fig. 1g, arrowhead) presumably derived by spontaneous mutations in *xylR* or P_{xyl} that resulted in constitutive expression of *murE* in the absence of inducer. By introducing a second copy of the *xylR* repressor gene and selection with high concentrations of penicillin G (PenG, an antibiotic that inhibits cell wall synthesis by inactivating penicillin-binding proteins), occasional colonies of pure L-form-like cells were obtained, which could be propagated indefinitely (Methods). The frequency at which L-form-like colonies were generated was lower than the rate of formation of xylose resistant rod-shaped mutants, which presumably arose by mutation of the P_{xyl} promoter or double *xylR* mutants. It therefore seems likely that one or more secondary mutations are needed to produce cells that can proliferate in this L-form-like state.

A strain generated by this method (Bs115 sup21) had many of the properties of L-forms made by classical methods (Fig. 1h, Supplementary Results and Discussion)[7,15]. Importantly, strain Bs115 sup21 was about 1,000 times more resistant to PenG than the wild-type strain (Supplementary Fig. 2), consistent with the idea that L-forms do not make peptidoglycan (PenG probably has non-specific toxic effects at very high concentrations). Strain Bs115 sup21 did not require PenG to grow in the L-form state and, in the absence of PenG and presence of *murE* inducer (xylose), only reverted to rod shape at low frequency. This suggests that at least one of the putative secondary mutations that it had acquired prevents it from making cell wall.

A mutation that predisposes for L-form proliferation

The frequency with which L-forms arose suggested that one or two mutations were needed to generate L-forms. To test this, we performed shotgun sequencing[16] of the complete genome of a freshly derived L-form strain (Bs115 sup23). Approximately 700 polymorphisms were identified by comparison of the sequence to that published in the SubtiList database[17]. Only four of these were also found to be different

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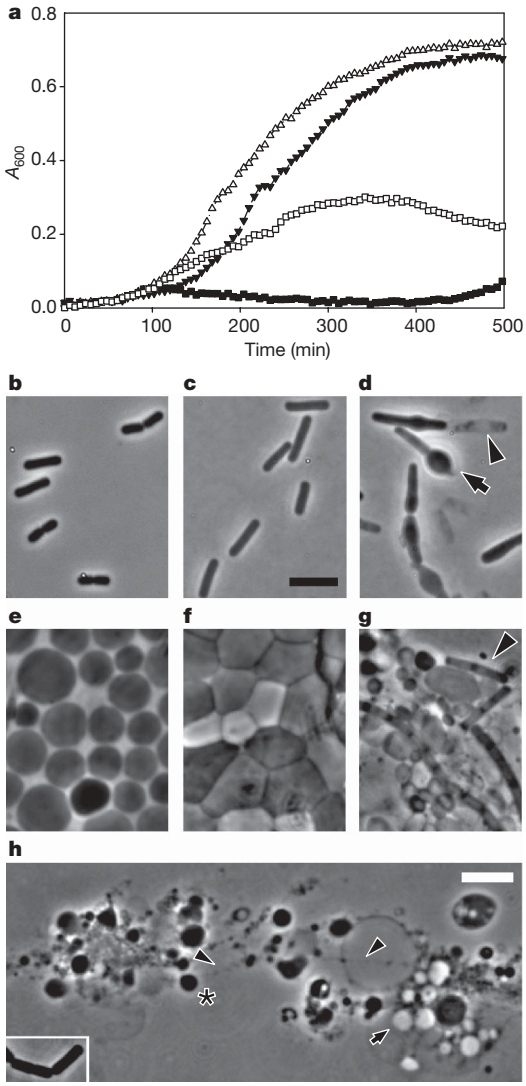

Figure 1 | Controlled generation of *B. subtilis* L-forms in cells with a repressible *murE* operon. **a**, The growth of the MurE depletion strain (M96) grown with *murE* expression (upright open triangle) and without *murE* expression either with (open square) or without Mg^{2+} (filled square) compared to the wild-type strain (inverted filled triangle). **b**–**h**, Phase contrast images of wild-type strain (**b**) and strain M96 expressing *murE* (**c**). Cells of strain M96 not expressing *murE* are bulged (**d**, arrow) and lytic (**d**, arrowhead). Cells of strain M96 not expressing *murE* form large spheroids in the presence of Mg^{2+} and sucrose (**e**) and tessellate when forced together (**f**). Cells of strain M96 not expressing *murE* grown in the presence of Mg^{2+}, sucrose and sloppy agar (**g**) have a similar appearance to L-form cells but can revert to rod-shaped cells (arrowhead). L-form strain Bs115 sup21 grown in liquid medium supplemented with $MgCl_2$, sucrose and penicillin G (**h**) are spherical and often connected by thin strands (arrow heads). Cells appeared either phase bright (arrow) or phase dark (asterisk). Inset, wild-type rods for comparison. Scale bar, 5 μm.

from a recently re-sequenced *B. subtilis* strain (A. Danchin, personal communication). Three of these four mutations turned out to be identical to substitutions in the immediate parent strain, Bs115. The one remaining polymorphism would generate a single amino acid substitution, D92E (GAT codon to GAA), in the *yqiD* gene. This gene encodes a protein of 272 amino acids, which is 45% identical to IspA of *Escherichia coli* (Supplementary Fig. 3). IspA catalyses the formation of farnesyl pyrophosphate in the isoprenoid synthetic pathway[18]. This pathway leads to the formation of several essential lipids, including bactoprenol, involved in peptidoglycan synthesis (and teichoic acids in *B. subtilis*). The D92E substitution (corresponding to D90 in the *E. coli* homologue, Supplementary Fig. 3) affects a conserved residue in the substrate-binding cavity of the enzyme[19] (Supplementary Fig. 4a, b) which is required for enzyme activity[20].

To test whether the mutation was related to the L-form phenotype, we constructed a strain with an extra copy of the mutant form of *ispA* driven by the IPTG (isopropyl-β-D-thiogalactoside)-dependent P_{spac} promoter. In rod-shaped cells, the integration did not significantly affect either growth rate or cell morphology, either in the presence or absence of IPTG (data not shown). We used the selective regime described above to test for L-form conversion in the presence of IPTG to express the mutant copy of *ispA* along with the wild-type copy. The results revealed about a 1,000-fold increase in the frequency of L-form-colony formation (average over three experiments, Supplementary Fig. 4c). In the typical experiment shown, the parent strain gave only three or four colonies when about a million cells were plated, whereas the mutant strain gave about 50 colonies from a 100-fold more dilute sample.

These results showed that a single point mutation in the *ispA* gene is sufficient to enable cells to grow without a wall. Although we do not yet understand the mechanism, it can be used to generate L-form strains at will, opening up a new era of research on these interesting cells and perhaps helping understanding of their role in pathogenesis and antibiotic resistance[21].

FtsZ-independent division

Although early biochemical experiments indicated that stable L-forms of Gram-positive bacteria do not synthesize detectable amounts of peptidoglycan[22–24], D'Ari and co-workers have recently reported that *E. coli* L-forms require residual peptidoglycan for division[6]. To test if this was also the case in our L-forms, we constructed a strain (Bs126) that was unable to synthesize D-alanine, which is an essential component of peptidoglycan. Rod-shaped bacteria required an exogenous source of D-alanine for growth, but an L-form strain derived from Bs126 grew without D-alanine (Supplementary Results and Discussion), suggesting that L-forms do not require peptidoglycan for division.

To begin investigating how L-forms divide without a cell wall, we constructed a strain that expressed an FtsZ–GFP fusion (strain Bs119 sup4). Fluorescence microscopy revealed spots and arcs of FtsZ–GFP, as well as complex networks of polymers (Fig. 2a). Unexpectedly, rings of FtsZ that might be supporting division were not evident. In the light of this observation, we tested whether FtsZ was actually required for L-form proliferation. To do so, we first used a strain in which the only copy of *ftsZ* was controlled by P_{spac}. As previously reported[25], when FtsZ was depleted in wild-type cells (strain BB11), division was completely inhibited and cells elongated for a few generations before lysing. Remarkably, when FtsZ was depleted in L-form strain Bs120 sup20, the growth rate was unaffected (Fig. 2b) and the strain could be indefinitely propagated without FtsZ expression. Western blot analysis confirmed that, when grown in the absence of inducer, FtsZ levels were barely detectable (Fig. 2b inset, lane 3).

To test if this residual amount of FtsZ was required for growth of the L-form, we attempted to make a strain with a complete deletion of *ftsZ* and *ftsA*, which form a two gene operon. The chromosomal copy of *ftsAZ* was deleted in the presence of an ectopic copy of the operon on the unstable plasmid, pLOSS[26] (Fig. 2c, lanes 5–7, Supplementary Results and Discussion). As FtsZ is essential in wild-type cells, only cells that keep the plasmid should survive. The presence or absence of pLOSS was assayed by PCR analysis and blue/white screening: colonies of plasmid containing cells appear blue on plates containing the β-galactosidase substrate X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside), because pLOSS expresses *lacZ* (encoding β-galactosidase; Supplementary Fig. 6a). As expected, in rod-shaped bacteria with *ftsAZ* deleted, all colonies were blue, demonstrating the requirement for pLOSS::*ftsAZ* (Supplementary Fig. 6c). However, an L-form strain with the same genotype gave only white colonies, suggesting that the strain had lost the plasmid and therefore that L-forms do not require FtsZ. Conventional PCR and quantitative

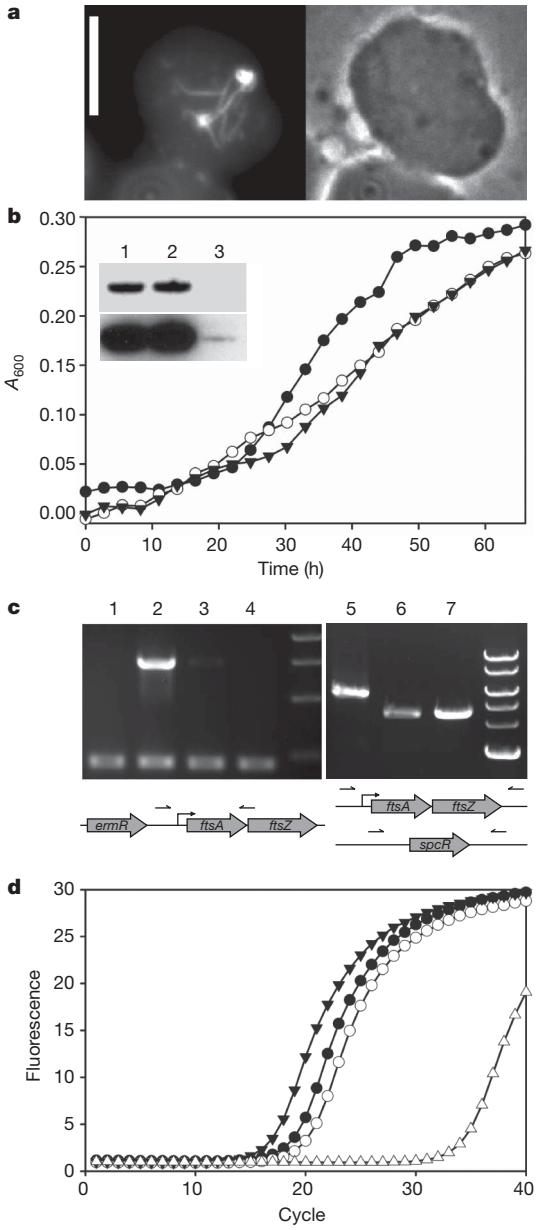

Figure 2 | L-forms do not require FtsZ for division. **a**, Localization of GFP–FtsZ in an L-form (strain Bs119 sup4). Left panel, GFP fluorescence; right panel, phase contrast image. Scale bar, 5 μm. **b**, Growth of the *ftsZ*$^+$ L-form (Bs115 sup21, filled circles) and the FtsZ depletion strain (Bs120 sup20) with (open circles) and without FtsZ expression (filled inverted triangles). Inset, western blot showing levels of FtsZ in *ftsZ*$^+$ L-forms (lane 1), from the FtsZ depletion strain with and without expression of FtsZ (lanes 2 and 3). Top panel, 15 s exposure; bottom panel, 5 min exposure. **c**, **d**, Control experiments showing that plasmid pLOSS::*ftsAZ* is lost in an L-form strain with a deletion of *ftsAZ* but not in a similar rod-shaped strain. **c**, Lane 1, PCR with oligonucleotides that bind on the plasmid backbone and in *ftsA* (diagram) amplified a 1.5 kb product from strain Bs161 (rod-form *ftsAZ*::*spc* pLOSS::*ftsAZ*, lane 2) but not from the wild-type strain (168, lane 1). A weak PCR product was amplified from chromosomal DNA of an L-form derived from Bs161 (lane 3) but no product was detected after this strain was plated for single colonies (lane 4). A control PCR reaction gave a 500 bp fragment from all chromosomal DNAs. PCR with oligonucleotides that bind either side of the *ftsAZ* operon (diagram) gave a 6 kb product with DNA from wild-type *B. subtilis* (lane 5) but only a shorter product with DNA from strain Bs161 or the L-form derived from Bs161 (lanes 6 and 7). **d**, Quantitative real-time PCR analysis of wild-type *B. subtilis* (circles) and the L-form strain derived from strain Bs161 (triangles). Panel shows the accumulation of PCR product (SYBR green fluorescence) during a 40 cycle PCR amplifying markers at the origin (filled symbols) and *ftsZ* loci (open symbols).

real time PCR confirmed that most rod-shaped cells kept pLOSS::*ftsAZ* but that in L-form cells the plasmid was either completely lost or present only in a very small sub-population of cells (about 1 plasmid per 250,000 chromosome origins; Fig. 2c, d; Supplementary Results and Discussion). Thus, the 'essential' *ftsZ* gene becomes non-essential in L-forms.

Proliferation by extrusion and resolution

To determine how L-forms proliferate without FtsZ, we developed conditions for long-term time-lapse imaging. L-form strains were grown in a glass-bottom microwell dish on the stage of the microscope at 30 °C (Methods). Observations of numerous fields over periods as long as 12 h (Supplementary Movie 1) showed that, although not all L-forms undergo growth, many cells, across the entire size range, showed a significant increase in size, indicating that they are metabolizing and synthesizing at least some cellular materials.

We observed several events that appeared to represent the normal mode of proliferation of L-form cells (Fig. 3). Initially, the more-or-less round L-form in Fig. 3a (Supplementary Movie 2) increased in size (arrowed, 10–320 min). It then went through a period when a series of shape perturbations occurred, characterized by the formation of transient blunt protrusions (370–410 min). These appeared and then retracted, and re-emerged from different points on the cell surface. Finally, a pseudopodium-like protrusion emerged and elongated over a period of about 70 min (Fig. 3a, arrowhead). This protrusion then resolved into about five more-or-less round bodies (540–560 min) that we believe represent progeny (see below). Although extrusion was slow, resolution can be rapid: in a separate experiment, a cell that had formed an extrusion was observed to resolve into three roughly spherical cells in less than one minute (Fig. 3d).

A similar extrusion-resolution process was also evident in smaller L-forms (<2 μm diameter); this sometimes gave rise to progeny of roughly equal size (Fig. 3b, Supplementary Movie 3), similarly to a previous report on division in *B. subtilis* L-forms[15]. This time-lapse imaging also demonstrated that at least some progeny are viable, because the two initial progeny both went on to undertake further division events. Note that one of these subsequent events gave rise to two roughly equal progeny, whereas the other was asymmetrical (Fig. 3b, 430 min, arrows and arrowheads, respectively).

The third kind of event we frequently observed generally involved large L-forms (3.5–6 μm diameter). In the example shown in Fig. 3c (Supplementary Movie 4), an L-form underwent a period of growth as a uniform sphere (10–270 min). This was followed by a period in which multiple, transient bulges occurred on the cell surface (Fig. 3c, 430–590 min, arrowheads). Finally, many small bodies erupted from a few sites on the cell surface and resolved into apparent progeny (Fig. 3c, 710–720 arrows).

Transmission electron micrographs (TEMs) of thin sections of L-forms revealed a heterogeneous population of cells, similar to those of stable L-forms reported previously[13,22]. Importantly, we often observed examples of cells apparently undergoing the proliferation events described above (Fig. 3e–g).

A strain that expressed a GFP-fusion to a DNA binding protein was used as a marker for the nucleoid. Time-lapse microscopy of this strain showed that cell proliferation was accompanied by many extrusion-resolution events (Supplementary Fig. 7). Multiple rounds of division resulted in the formation of a micro-colony, suggesting that progeny were viable. Every large cell-like object in the colony had a GFP signal, showing that the proliferative events are accompanied by segregation of DNA (Supplementary Results and Discussion).

Conclusions and perspective

These experiments were originally prompted by the question of whether Z-ring constriction requires concomitant cell wall synthesis. We noticed that although FtsZ forms polymers in L-forms, it does not form significant numbers of rings, nor is there any sign of FtsZ-associated constriction. The results therefore support a model in which the constriction of the Z-rings is dependent on wall synthesis.

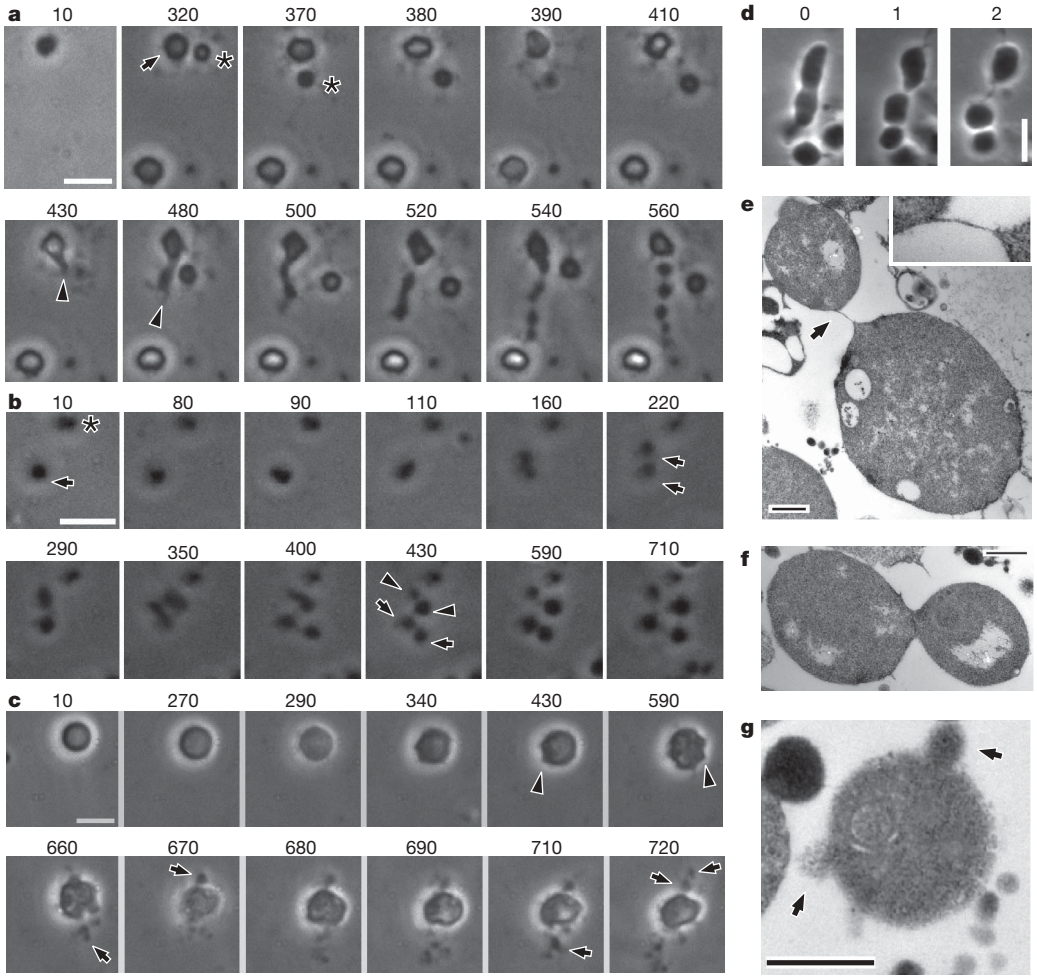

Figure 3 | Novel mechanisms of proliferation revealed by time-lapse imaging of L-forms. **a**–**c**, Still images from Supplementary Movies 2–4, with times indicated in min. Scale bars, 5 μm. **a**, The cell marked with an arrow (at 320 min) begins to form a protrusion at 430 min (arrow head) which resolves into a string of six cells visible at 560 min. Note that another cell (marked with an asterisk at 320 min) drifts into the field of view and was not the result of a proliferative event during the movie. **b**, The cell marked with an arrow (at 10 min) divides after 160 min to produce two cells (220 min, arrows), which divide again after 350 min to give symmetrical progeny (430 min, arrows) and asymmetrical progeny (430 min, arrowheads). Another cell (marked with an asterisk at 10 min) does not divide during the time course. **c**, Surface blebs emerge from the cell (430–590 min, arrowheads), and later, multiple small progeny are extruded (arrows). **d**, An elongated cell at 0 min resolves into three roughly spherical cells after 1 min. **e**–**g**, Transmission electron micrographs of dividing L-forms. **e**, Two cells connected by a thin strand (arrow, and inset at 3× magnification). **f**, A cell apparently at a late stage of a resolution event. **g**, A cell with surface blebs and possible progeny (arrows). Scale bars, 500 nm.

However, it remains possible that the transition to the L-form state may involve other changes that are incompatible with Z-ring constriction. Nevertheless, *ftsZ* could be deleted in L-forms, showing that although it is normally essential in many bacteria, it becomes non-essential in cells growing without a wall.

Prokaryotes that do not have peptidoglycan in their envelopes include members of the class Mollicutes (including the major genera *Mycoplasma*, *Ureaplasma*, *Acholeplasma*, *Spiroplasma* and *Phytoplasma*[27]), the order *Planctomycetales*[28] and the archaea[29]. Of these, some also lack FtsZ, including the *Phytoplasma*, and the archaeal phylum crenarchaeota[30]. Little is known of the replication of these organisms, but the unusual mode of proliferation we have uncovered for *B. subtilis* L-forms may be of relevance to them.

In principle, it is difficult to envisage how extrusion-resolution could occur in the absence of an active force generation system within the cells[31]. Therefore, it seems likely that some kind of cytoskeletal system is involved. FtsZ appears not to be required, so the most likely candidates would be one or more of the MreB (Actin) homologues, of which *B. subtilis* has three: MreB[32], Mbl[33] and MreBH[34]. Alternatively, extrusion could be driven by an active chromosome segregation system. Extrusion of a nucleoid followed by collapse and resealing of the membrane could explain the resolution process we observed. The molecular details underlying this process, reminiscent of pseudopod production in higher cells, will be an important area for future work.

Finally, we speculate that the novel mechanism of proliferation we have discovered has implications for the evolution of early forms of life. Genes for the synthesis of peptidoglycan are found throughout all major branches of the bacterial kingdom[35], suggesting that the last common ancestor already had a wall. However, this cell is likely to have been derived from an earlier wall-less progenitor. We propose that an extrusion-resolution mode of proliferation may have been used in these progenitor cells. Later, when the wall evolved, elements of this mechanism were used to drive segregation along the long axis of the cylindrical tube formed by the rigid wall. The system may have been retained for occasional use when the wall was damaged, for example, during antibiotic 'warfare' between competing microorganisms. The Mollicutes may represent specialized cells that have readopted this mode of replication as part of their adaptation to a life style intimately associated with modern higher eukaryotes.

METHODS SUMMARY

Reproducible method for generation of L-form bacteria. Strain Bs115, or a derivative of that strain, was grown to late exponential phase in rich medium supplemented with 20 mM $MgCl_2$, 0.5 M sucrose and buffered with 20 mM

maleic acid (this supplement is referred to as MSM) and 0.5% w/v xylose. Cells were washed once in MSM in H_2O. 100 µl of cells was added to 5 ml of nutrient broth (NB, Oxoid) with MSM and molten 1% w/v type VII low gelling temperature agarose ('soft agar', Sigma) that had been cooled to 37 °C, then spread on top of a nutrient agar (NA, Oxoid) plate supplemented with MSM, but without xylose. Plates were inverted and incubated at 30 °C overnight in an airtight container. After 12–14 h incubation, 1 cm^2 blocks of sloppy agarose that did not contain colonies of rod-shape cells were spread on fresh NA plates supplemented with MSM and 200 µg ml^{-1} PenG (Sigma). Atypical colonies containing L-form-like cells formed after 3–4 days.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Author Contributions M.L. and J.E. designed the research and wrote the manuscript. M.L. performed the experiments. P.D.-C. constructed plasmids and strains and contributed to discussions. J.M.C. performed the genome sequencing. R.A.D. constructed strains, and contributed to discussions and the design of the research. All authors commented on the manuscript.

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METHODS

Growth of strains. For growth in liquid media, strains were grown in nutrient broth (NB, Oxoid), or brain heart infusion/yeast extract (BHIYE: 37 g l^{-1} brain heart infusion with 5 g l^{-1} yeast extract, both from Oxoid). For growth on solid media, strains were grown on nutrient agar (NA, Oxoid) or in semi-solid media by adding 10 g l^{-1} type VII low gelling temperature agarose (Sigma) to liquid media. Where necessary, media were supplemented with 0.5% xylose, 1 mM IPTG, 10 μg ml^{-1} D-alanine or with 20 mM $MgCl_2$, 0.5 M sucrose and 20 mM maleic acid (MSM). As we were unsuccessful in storing L-form strains frozen in glycerol, they were propagated continuously on NA/MSM plates supplemented with 200 μg ml^{-1} penicillin G (PenG, Sigma), and re-streaked onto fresh plates every 3–4 days. Liquid cultures were initiated in NB/MSM supplemented with 200 μg ml^{-1} PenG, grown at 30 °C without shaking. To ensure adequate aeration in these conditions, the ratio of media volume:flask volume was below 1:10.

Information about the construction and genotypes of strains and plasmids are presented in Supplementary Tables 1 and 2, respectively.

Growth curves. L-form strains were inoculated into NB/MSM media supplemented with 200 μg ml^{-1} PenG. The culture was divided into portions to which the appropriate inducer or supplement was added. The culture was then aliquoted into a 96 well microtitre plate with a blank of media only. Growth of the culture at 30 °C was quantified by absorbance readings at 600 nm (A_{600}). Readings were taken every 2.75 h by a microtitre plate reader (FluoStar Galaxy, BMG Lab Technologies) with 5 min shaking before each reading. Growth curves were plotted as the average of 7 repeats.

Time-lapse microscopy. Successful time-lapse microscopy required a number of modifications to the protocols typically used for normal bacteria, mainly reflecting the slow growth rate of L-forms. L-forms were grown in 35 mm glass-bottom microwell dishes (MatTek) and visualized through the coverslip mounted over the hole in the bottom of the dish. The top surface of the coverslip was treated with 0.1% poly-lysine w/v in H_2O (Sigma) for 5 min then washed with NB/MSM. A 200 μl sample of a culture of L-forms grown in NB/MSM overnight at 30 °C was allowed to adhere to the surface of the coverslip for 10 min. Non-adherent cells were washed away with NB/MSM. 2 ml of NB/MSM was then added to the microwell dish to provide a reservoir of medium. The dish was then sealed to make an airtight chamber. To ensure that cells were not subjected to an osmotic shock, NB/MSM from the overnight culture, which had been cleared of cells by centrifugation, was used throughout the mounting procedure. The microwell dish was placed on the microscope stage and allowed to acclimatize to 30 °C for 30 min.

Electron microscopy. Samples were prepared by the Electron Microscopy Research Service of Newcastle University. Briefly, cells were fixed overnight in 2% glutaraldehyde in Sorenson's phosphate buffer (TAAB Laboratory Equipment), pH 7.4, then in 1% osmium tetroxide (Agar Scientific) for 1 h. Samples were then dehydrated in an acetone graded series before being impregnated with a graded series of epoxy resin (TAAB Laboratory Equipment) in acetone and finally embedded in 100% resin and set at 60 °C for 24 h. Cells were sectioned and counter stained with 2% uranyl acetate and lead citrate (Leica) before being imaged on a Philips CM100 Compustage Transmission Electron Microscope (FEI) with a AMT CCD camera (Deben).

Quantitative real time PCR. DNA was extracted from an exponentially growing culture of strain Bs161 (*ftsAZ*::*spc* pLOSS::*ftsAZ*) and a stationary phase culture of L-forms derived from the same strain, both were grown without selection for the plasmid. Primer pairs GATCAATCGGGGAAAGTGTG, GTAGGGCCTGTGGATTTGTG and TCCATATCCTCGCTCCTACG, ATTCTGCTGATGTGCAATGG were used to amplify the origin and terminus regions respectively, while CATTAACAGTCGGCGTTGTG, ATCCACCGCTTCTTTCATTG were used for *ftsZ*. PCR reactions were performed using Power SYBR Green PCR Master Mix (Applied Biosystems) and quantitative real time PCR was performed in a LightCycler 480 Instrument (Roche), in accordance with the manufacturer's instructions.

DNA sequencing. Genomic DNA was extracted using phenol:chloroform, and further purified using the Qiagen Genomic DNA Purification kit. Sequencing was performed using a Roche FLX Genome Sequencer. Preparation of the single-stranded template DNA library and shotgun sequencing were carried out as described by the manufacturer. Individual sequencing reads were assembled into 47 contigs using GS Mapper software (Roche) with the *B. subtilis* genomes from Subtilist[17] and A. Danchin (personal communication) as a reference sequence. Polymorphisms were filtered using an algorithm written by D. Swan of the Bioinformatics Support Unit, Newcastle University.