MICROBIOLOGY SOCIETY

L form bacteria growth in low-osmolality medium

Masaki Osawa*,† and Harold P. Erickson

Abstract

L form bacteria do not have a cell wall and are thought to require medium of high osmolality for survival and growth. In this study we tested whether L forms can adapt to growth in lower osmolality medium. We first tested the *Escherichia coli* L form NC-7, generated in 1987 by Onoda following heavy mutagenesis. We started with growth in osmoprotective medium (~ 764 mOsm kg^{-1}) and diluted it stepwise into medium of lower osmolality. At each step the cells were given up to 10 days to adapt and begin growing, during which they apparently acquired multiple new mutations. We eventually obtained a strain that could grow in LB containing only 34 mM NaCl, 137 mOsm kg^{-1} total. NC-7 showed a variety of morphologies including spherical, angular and cylindrical cells. Some cells extruded a bud that appeared to be the outer membrane enclosing an enlarged peri-plasm. Additional evidence for an outer membrane was sensitivity of the cells to the compound CHIR-090, which blocks the LPS pathway, and to EDTA which chelates Mg that may stabilize and rigidify the LPS in the outer membrane. We suggest that the mechanical rigidity of the outer membrane enables the angular shapes and provides some resistance to turgor in the low-osmolality media. Interestingly, cells that had an elongated shape underwent division shortly after addition of EDTA, suggesting that reducing the rigidity of the outer membrane under some turgor pressure induces division before lysis occurs. We then tested a well-characterized L form from *Bacillus subtilis*. L form strain LR-2L grew well with sucrose at 1246 and 791 mOsm kg^{-1}. It survived when diluted directly into 440 mOsm kg^{-1} but grew poorly, achieving only 1/10 to 1/5 the density. The *B. subtilis* L form apparently adapted to this direct dilution by rapidly reducing cytoplasmic osmolality.

INTRODUCTION

Many bacterial species can develop a state called an L form, which has lost the peptidoglycan cell wall. L forms generally require an osmoprotective medium for survival and growth. The existence of L form bacteria has been known for a long time, although with ambiguity as to their nature [1]. However, a very clear and simple definition for a *Bacillus subtilis* L form was reported in a series of recent papers [2–4]. A primary requirement to produce this L form is inhibition of the pepti-doglycan synthesis pathway. This could be achieved either by mutation or by chemical inhibition of enzymes in the pepti-doglycan synthesis pathway. This requirement is direct and obvious because L form is defined as 'wall-less bacteria'. Muta-tions in two additional pathways were required to obtain the *B. subtilis* L form. One led to increased membrane synthesis, which is probably important for budding-type cell division [3]. The second counteracted reactive oxygen species, which

are generated in excess by the electron transport pathway in wall-less bacteria [5].

The division of L form bacteria does not depend on FtsZ, which is the primary cytoskeletal protein for the normal bacterial division machinery. As spherical L forms grow to a larger volume than the original walled bacteria, the volume/surface ratio increases. If the rate of membrane production is constant, the membrane will be in excess of what is needed for the surface of the sphere. The excess membrane should bend outward or inward, producing protrusions or vacuoles. It is thought that this type of shape change is the driving force for the division for L forms [3]. As a result, divisions occur at almost random shape and size.

L forms may be more complicated for Gram-negative bacteria because of the outer membrane. Gram-negative L forms have been reported with and without an outer membrane [6]. However another study reported that an L form that lacked a

double membrane, as seen by thin-section electron microscopy (EM), had LPS, a component of the outer membrane, in its membrane fraction [7]. Reviewing the older literature we suggest that thin-section EM can provide convincing evidence for the existence of an outer membrane, but not for its absence. When the two membranes are separated by a wide periplasmic space the outer membrane is clear. However, when there is no obvious periplasmic space, thin-section EM generally cannot distinguish a single inner membrane from two membranes closely pressed together.

Here we used a heavily mutated *Escherichia coli* L form, strain NC-7, originally produced by Onoda *et al.* [8]. NC-7 has amorphous shapes that become spherical after treatment with EDTA to remove divalent cations from the culture medium [9]. Although thin-section EM showed no obvious outer membrane in the original study of NC-7 [8], we provide evidence that it does have an outer membrane, and that this is important for its shape and stability.

We have undertaken a new study to show that the Onoda L form can adapt to growth in medium of reduced osmolality. We then repeated the study for the well-characterized *B. subtilis* L form and found that it can also adapt to direct dilution into reduced osmolality medium, although not as low as *E. coli*.

METHODS

Bacterial strains and culture

The *E. coli* L form strain NC-7 was donated by Dr T. Onoda (strains are listed in Table S1, available in the online version of this article). NC-7 was cultured in our standard osmoprotective medium MLB (1 % peptone, 0.5 % yeast extract, 340 mM NaCl, 1 mM CaCl$_2$, 30 mM glucose, 25 mM MOPS pH 7.2, 100 U ml^{-1} penicillin G) (all media are detailed in Table 1). To test the ability of NC-7 to adapt to lower osmolality, the NaCl, which is the osmoticum in MLB, was reduced stepwise from 340 mM to 272, 204, 136, 68, 34 and 0 mM NaCl. NC-7 was diluted 100-fold into the lower osmolality medium, for example from 340 to 272 mM NaCl, to see if it could grow. Once NC-7 growth was established, it was diluted into the next lower osmolality medium. These adaptations were repeated with regular LB [1% tryptone, 0.5% yeast extract, 1 % (170 mM) NaCl] supplemented with 100 U ml^{-1} penicillin G. Initially NC-7 that was previously adapted in 130 mM NaCl MLB was diluted into LB (170 mM NaCl). When growth was established, the adapted NC-7 was diluted sequentially to lower osmolality LB, where NaCl concentrations were 136, 102, 68, 51 and 34 mM. In each case, when growth was established the NC-7 was diluted 1:100 into the next lower osmolality.

Cell growth in each medium was first measured by turbidity as the optical density at 600 nm (OD$_{600}$). Because NC-7 strains in different osmolality media have different shapes and size, the OD$_{600}$ measurements may not be accurate for comparison. To confirm the OD$_{600}$ results, we developed a method for cell density measurement using Coomassie staining. To

Table 1. The composition and osmolalities of media used herein as measured by a vapour pressure osmometer. The measured osmolalities were within 5–15 % of the estimated values used in the literature [10–12] (*BD Bionutrients Technical Manual*, 3rd edition revised) (Difco and BBL manual, *Manual of microbiological culture media*, 2nd edition)

Medium	Composition	Osmolality (mOsm kg^{-1})
LB (Miller)	1 % tryptone, 0.5 % yeast extract, 100 U ml^{-1} penicillin G, 170 mM NaCl	410
LB 68	Same as LB except 68 mM NaCl	212
LB 51	Same as LB except 51 mM NaCl	171
LB 34	Same as LB except 34 mM NaCl	137
MLB	1 % peptone, 0.5 % yeast extract, 1 mM CaCl$_2$, 30 mM glucose, 25 mM MOPS pH 7.2, 100 U ml^{-1} penicillin G, 340 mM NaCl	764
MLB 272	Same as MLB except 272 mM NaCl	648
MLB 204	Same as MLB except 204 mM NaCl	504
MLB 136	Same as MLB except 136 mM NaCl	388
MLB 68	Same as MLB except 68 mM NaCl	256
MLB 34	Same as MLB except 34 mM NaCl	187
MLB 0	Same as MLB except 0 mM NaCl	132
2xPAB/SMM 500	2xPAB: 7 % Difco antibiotic medium No. 3; SMM: 20 mM sodium maleate, 20 mM MgCl$_2$ pH 6.5, 1 µg ml^{-1} benzamide, 500 mM sucrose	1246
2xPAB/SMM 400	Same as 2xPAB/SMM except 400 mM sucrose	1077
2xPAB/SMM 300	Same as 2xPAB/SMM except 300 mM sucrose	948
2xPAB/SMM 200	Same as 2xPAB/SMM except 200 mM sucrose	791
2xPAB/SMM 100	Same as 2xPAB/SMM except 100 mM sucrose	660
2xPAB/ SMM 0	Same as 2xPAB/SMM except 0 mM sucrose	548
LB +10 mM MgCl$_2$	1 % tryptone, 0.5 % yeast extract, 170 mM NaCl, 10 mM MgCl$_2$, 1 µg ml^{-1} benzamide	440

avoid lysis upon centrifugation, L forms were fixed with 4 % paraformaldehyde for 1 h at room temperature. The culture was then centrifuged at 13 400 r.p.m. for 60 s (Eppendorf minispin) and L forms were washed with PBS followed by water. After carefully removing water, 200 µl of Commassie staining solution (1 g Commassie brilliant blue R-250 in 450 ml methanol, 450 ml glacial acetic acid and 100 ml H$_2$O) was added and left for 1 h to stain the pellet. The stained pellets were washed twice with water. After carefully removing the water, Coomassie dye was resolubilized with 100 µl destainer (methanol/acetic acid=9 : 1, v/v) for 24 h at room temperature. The resolubilized Commassie dye was collected from the supernatant and the dye concentration was measured by absorption at 590 nm.

B. subtilis strain LR-2 was donated by Dr J. Errington (Table S1). A *B. subtilis* L form was generated as described by Mercier *et al.* [3]. Briefly, LR-2 was cultured in LB supplemented with 0.5 % xylose for expression of MurE, 10 µM tetracycline and 5 µg chloramphenicol ml^{-1} until the OD$_{600}$ reached 0.2. The cells were pelleted and resuspended in osmoprotective medium 2xPAB/SMM (2xPAB: 7 % Difco antibiotic medium No. 3; SMM: 500 mM sucrose, 20 mM MgCl$_2$, 20 mM sodium maleate, pH 6.5) supplemented with 0.5 mg lysozyme ml^{-1} and 1 µg benzamide ml^{-1} (an inhibitor of FtsZ). The cells were cultured for 1 h at 37 °C in a shaker and then diluted 1000-fold in 2xPAB/SMM supplemented with 1 µg benzamide ml^{-1}. The diluted cells were cultured without shaking at room temperature and grew at the bottom of the tubes. The L form generated from LR-2 is referred to as LR-2L.

To check the ability of LR-2L to adapt to growth at lower osmolality, sucrose, which is the osmoticum in 2xPAB/SMM, was reduced stepwise from 500 mM to 400, 300, 200, 100 and 0 mM as described above for NaCl reduction for NC-7. Growth of LR-2L was also tested in LB, LB + 10 mM MgCl$_2$ and LB with NaCl increased by 15 mM (adjusting the osmolality to equal that of LB + 10 mM MgCl$_2$). These LB-based media contain benzamide at 1 µg ml^{-1}.

The osmolality of each medium was measured using a Wescor Inc 5600 vapour pressure osmometer, and values are reported in Table 1.

Transformation

The expression of membrane-targeted FtsZ (FtsZ-YFP-mts) [13] and fully functional FtsZ-YFP [14] was tested by transforming NC-7 with a pJSB plasmid. For expression of FtsZ-YFP, we switched antibiotic resistance of the pJSB plasmid from chloramphenicol to kanamycin. The original pJSB plasmid including FtsZ-YFP was entirely amplified by PCR except the chloramphenicol gene, and the linear PCR product was ligated with the kanamycin-resistant gene plus its promoter from plasmid pEGFP-C1 (Clontech). NC-7 that was adapted to MLB with 170 mM NaCl was transformed with pJSB as previously described for the transformation of the *B. subtilis* L form [5], with some modifications. NC-7 was cultured in a 50 ml conical tube with 5 ml MLB medium without shaking at room temperature. Then, 300 µl of strain NC-7 growing at the bottom of the tube was collected and transferred to a 15 ml conical tube. After gently mixing with 150 µl PAB/SMM, 2 µg plasmid was added. The transformation reaction was started with addition of 450 µl of 40 % PEG (molecular weight 6000 Da) in SMM, and the reaction was stopped after 2 min by the addition of 1.5 ml PAB/SMM. The mixture was left for 3 h at room temperature and then directly plated on an MLB agar plate supplemented with chloramphenicol for pJSB FtsZ-YFP-mts or kanamycin for pJSBKn_FtsZ-YFP.

Microscopy

Differential interference contrast (DIC) and fluorescence microscopy images of bacteria and cells were obtained with a Zeiss Axiophot microscope with a 100× (NA 1.3) or 40× (NA 1.3) objective lens and a CCD camera (CoolSNAP HQ; Roper). The FM4-64 signal was captured through a rhodamine filter cube (Chroma BP546 FT580 BP590), and yellow fluorescent protein (YFP) with a YFP filter cube (Chroma BP500/20 515 BP535/30). The phase contrast images were acquired with a Zeiss Axiophot microscope with a 100×, NA 1.4 objective. The cell shapes and sizes were evalutated by ImageJ.

RESULTS

E. coli L form NC-7 can adapt to low osmolarity conditions

We tested the *E. coli* L form NC-7, which was created by Onoda following heavy mutation [8], for its ability to adapt to growth in low-osmolarity medium. Our standard culture medium (MLB) for NC-7 contains 340 mM NaCl as the osmoticum (Table 1). The shape and size of the L form in MLB are variable (Fig. 1a). Although we occasionally found rod-like cells of various sizes, these probably do not have a cell wall because (1) penicillin G was added to all media for NC-7 growth, and (2) rod-like cells were always rare and never dominated in the population even in lower osmolality medium (Fig. 1b, c). The adaptation of NC-7 to lower osmolality was attained by stepwise decreases of NaCl concentration. In each step, NaCl was decreased by 34 mM and once growth was established at a given osmolality, NC-7 cells were diluted 100-fold into new lower osmolality MLB. Establishing growth took up to 10 days, but the actual time varied for the different steps. We found that with this stepwise protocol NC-7 could adapt to growth in MLB even in the absence of NaCl (Fig. 1c, d).

NC-7 in lower osmolality medium showed morphological changes. There was an increase in cell volume especially in MLB lacking NaCl. Some cells showed a vacuole-like structure, and some showed a protrusion of a low-contrast bleb that appeared to be surrounded by a membrane in DIC (Fig. 1c, d) and phase contrast images (Fig. 1i, j). We suggest that this membrane, which stains with the membrane dye FM4-64 (Fig. 1k), is the outer membrane, and the enclosed space is equivalent to the periplasmic space. The low contrast of the bleb is attributed to the absence of cytoplasmic proteins and nucleic acid in the periplasmic space. Although the original study of NC-7 did not detect an outer membrane in thin-section EM, we believe that a tightly opposed inner and outer membrane could not be excluded.

Because Onoda *et al.* found that supplementation of Ca ions in the medium was important for NC-7 growth, we tested LB-based medium, which does not have added Ca or Mg ions. NC-7 in MLB with 136 mM NaCl was transferred to standard LB that contained 170 mM NaCl, and the NaCl concentration was reduced stepwise until NC-7 was not able
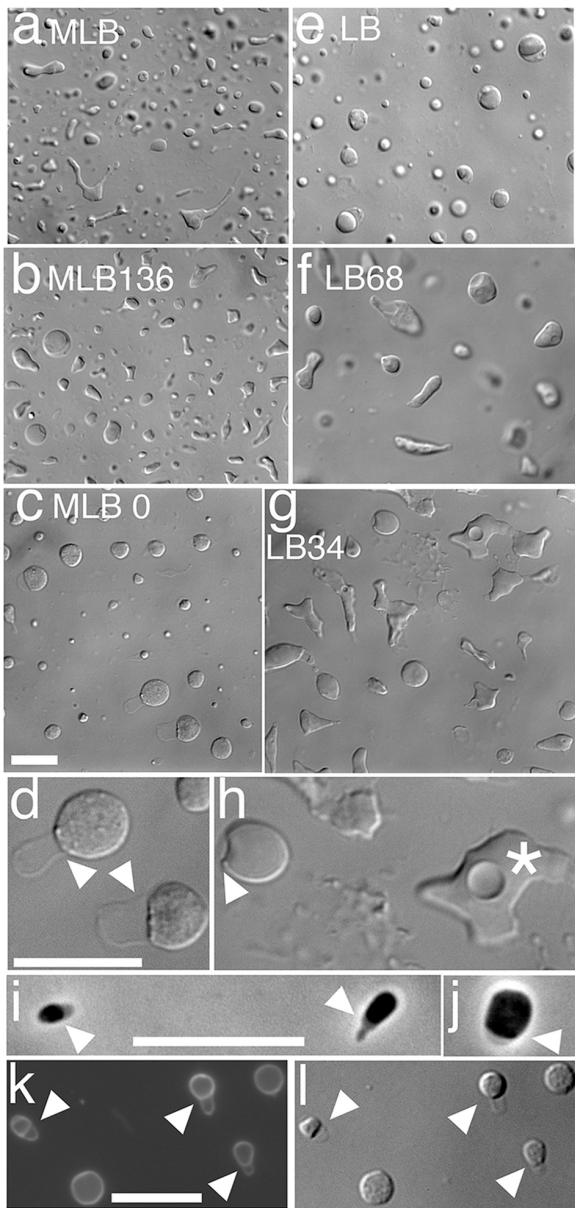
Fig. 1. Morphology of cells of strain NC-7 growing in low-osmolality conditions. NC-7 growing in MLB with 340 mM NaCl (a), 136 mM NaCl (b) and zero NaCl (c, d, i, j, k, l). NC-7 growing in LB with 170 mM NaCl (e), 68 mM NaCl (f) and 34 mM NaCl (g, h). All are DIC microscopy images except (i)–(k). (d) and (h) are magnified from (c) and (g). (i) and (j) are phase contrast images. (k) is a fluorescence image where the membrane was stained with FM4-64 dye. (l) shows the DIC image of (k). Arrowheads show the outer membrane separate from the inner membrane. The outer membrane is identified as the low-contrast projection in (d), (i) and (j). The asterisk highlights a vacuole-like structure in the cytoplasm. Bars, 10 µm. (a)–(c) and (e)–(g) are at the same magnification; (d) and (h) are at the same magnification; (i) and (j) are at the same magnification.

we obtained a strain that survived in LB with 34 mM NaCl (osmolality 137 mOsm kg^{-1}) (Fig. 1g). This difference suggests that these strains acquired mutations to adapt to low osmolality. Therefore, these strains are referred to as NC-7/51 and NC-7/34, respectively. In general, the morphology of the NC-7 grown in low-osmolality LB medium was similar to the original NC-7 in MLB medium (Fig. 1e, f, g). The rod-shaped L forms were also observed in LB medium, but again they should not have a peptidoglycan wall because the LB-based medium contained penicillin G. Also NC-7/34 does not have a functional *murE* gene, which is essential to produce the cell wall (Table S3). In the LB-based medium, some cells had a larger volume than those in MLB medium, probably due to a lower division frequency. NC-7/34, in particular, is much larger and has an angular shape. The size and shapes were quantitated as detailed in Figs S1 and S3.

NC-7 in low-osmolality medium grew more slowly and reached a lower cell density than at higher osmolality. The NC-7 was cultured without shaking at room temperature, so cells settled and grew at the bottom of the tube. To quantify growth we initially agitated the cells and measured the turbidity. The maximum turbidities (OD$_{600}$) in a series of culture media were: MLB (340 mM NaCl), 0.46; MLB (136 mM NaCl), 0.42; MLB (0 mM NaCl), 0.17; LB (170 mM NaCl), 0.40; LB (68 mM NaCl), 0.35; LB (51 mM NaCl), 0.37; and LB (34 mM NaCl), 0.26 . A quantitative interpretation of the OD$_{600}$ values is compromised, however, because the shape and size of L forms are not constant. Because L forms growing in low-osmolality medium tend to be larger, as shown in Fig. S1, the OD$_{600}$ would overestimate their cell number.

We therefore developed a method to measure and compare the growth of each strain. Quantification of the L form mass was complicated because these forms lysed during centrifugation. We therefore fixed the cells with paraformaldehyde prior to centrifugation and washing the pellets. The fixed cell pellets were stained with Coomassie brilliant blue, washed and incubated with destainer to resolubilize the Coomassie blue. The Coomassie assays for various L form lines in different media are given Fig. S2a. The maximum cell masses assayed by Coomassie were generally consistent with those from the turbidity assay.

The growth rate for NC-7 indicated by the Coomassie assay (Fig. S2) was fastest for MLB 340–136 mM NaCl, slower for LB and very slow for MLB 0 NaCl.

NC-7 and the derivative adapted to low-osmolarity growth have multiple mutations

When NC-7 cultured in MLB was directly diluted 100-fold in MLB 0 (skipping stepwise adaptations), it was not able to grow. Likewise NC-7 adapted in LB 170 mM NaCl did not grow when diluted directly in LB 51 (51 mM NaCl). Therefore, we hypothesized that the adaptations were accomplished by an accumulation of mutations.

to survive. NC-7 could adapt and grow in LB containing 51 mM NaCl, which has an osmolality of 171 mOsm kg^{-1}. In the first trial, this was the lowest osmolality achieved for adaption. However, when we repeated this procedure,

The original L form NC-7 was selected following heavy mutagenesis with nitrosoguanidine [8], and we judged that additional mutations occurred during our selection for growth at low osmolality. We therefore used Illumina whole genome sequencing of our laboratory NC-7 strain (which had undergone several rounds of growth for other experiments) and the derivative NC-7/34 adapted for low-osmolality growth. Our original NC-7 strain has 264 mutations in the genome, compared to the K-12 substrain W3110 (NC_007779.1). The mutations comprise four insertions/deletions and 260 SNPs including 33 intergenic, 73 synonymous and 154 non-synonymous mutations (Table S2). Some mutations are in important genes such as *ftsA* (cell division molecule), *lpxB* (lipid A synthesis), *mscS* (mechanosensitive ion channel), *mutT* (DNA repair) and *mug* (DNA repair). These mutations are SNPs which probably reduce the activity of some of these molecules. FtsA is an essential molecule for bacterial division, but because FtsZ is not responsible for NC-7 division, FtsA should be dispensable. LpxB is essential for lipid A synthesis. Because we conclude here that NC-7 retains its outer membrane, this SNP should not completely remove LpxB activity but may reduce it. The DNA repair mutations suggest that the NC-7 genome may be unstable and prone to further mutations. Consistent with this, NC-7/34 acquired many mutations during the process of adaptation. The mutations in NC-7/34 relative to our laboratory NC-7 strain comprise 21 insertions/deletions and 324 SNPs including 36 intergenic, 94 synonymous and 194 non-synonymous mutations (Table S3). Mutations in genes important for cell division also increased in NC-7/34. For example, FtsI and MurE, both essential for cell-wall synthesis, are probably not functional in NC-7/34 because of frameshifts. This confirms our interpretation that NC-7/34 can survive and form various shapes, including the rod shape, using primarily its outer membrane.

Role of the outer membrane of NC-7 in maintaining cell shape and division

How can the L form maintain the rod and angular shapes in low osmotic medium without a cell wall? Our DIC, phase contrast and fluorescence microscopy results (Fig. 1) suggest the presence of two cell membranes. Because Onoda *et al.* showed that EDTA treatment changed NC-7 from an irregular angular shape to spherical [9], we hypothesized that the outer membrane, including LPS and associated divalent cations, may be important to provide rigidity to resist turgor pressure and maintain the angular shapes. To test the importance of the outer membrane, we used the inhibitor CHIR-090, which acts as an antibiotic and kills Gram-negative bacteria. CHIR-090 specifically blocks the LPS synthase LpxC [15], and thus disrupts the outer membrane. As shown in Fig. 2(b), a filter paper soaked with 20 µg CHIR-090 inhibited growth of *E. coli* JM109, a normal walled K-12 strain used here as a control. CHIR-090 also strongly inhibited NC-7 (Fig. 2a); the larger circle of inhibition is probably due to slower growth of NC-7, allowing larger diffusion of CHIR-090 before the lawn
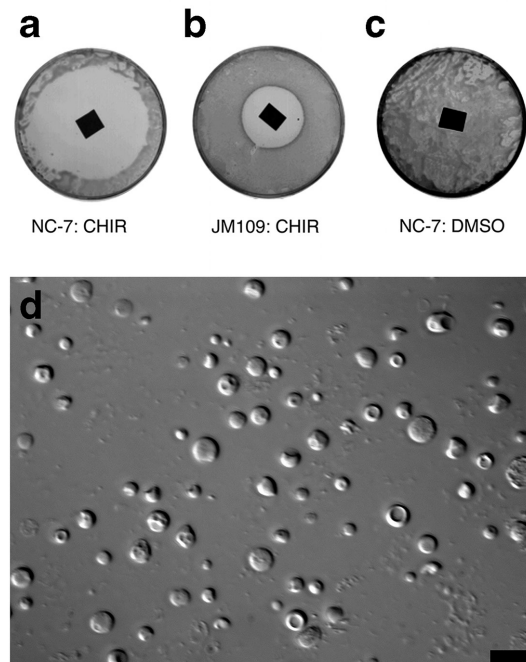
Fig. 2. Effect of CHIR-090 on the growth and morphology of NC-7. NC-7 (a, c) and JM109 (b, a normal K12 strain) were heavily plated on MLB agar plates to generate a lawn of cells. Then, 20 µg of CHIR-090 was applied to a filter paper square and placed on the agar plates immediately after plating the cells. Plates were imaged after 1 day of growth. (c) Instead of CHIR-090, DMSO was applied as a negative control. (d) DIC image of NC-7 was captured 1 day after CHIR-090 was added at 10 µg ml⁻¹ to NC-7 growing in MLB medium. The cells have lost their angular shapes and are mostly round (compare with Fig. 1a). Bar, 10 µm.

appears. Fig. 2(d) shows that CHIR-090 added to a suspension culture in MLB changed the morphology of the NC-7 from pleomorphic and angular to spherical. This clear shape change is confirmed quantitatively in Fig. S3. This suggests that the angular morphology requires rigidity of the outer membrane (Fig. 3d).

Onoda *et al.* showed that 1 mM EGTA added to their NaPY medium caused strain NC-7 to change from irregular angular shapes to spherical [9]. The EGTA specifically chelated the 0.12 mM Ca in their medium and 'ignored' the larger concentrations of Fe, Zn and Mn. The only divalent cation in our MLB is 1 mM Ca, so we chelated this by adding 10 mM EDTA. In the osmoprotective MLB medium this caused the cells to become spherical (Fig. 3a), as reported by Onoda *et al.* The clear difference is confirmed in Fig. S3. We then tested the effect of adding EDTA to NC-7/34 growing in the low-osmolality LB medium, which probably has some Ca and Mg from the tryptone and yeast extracts [16]. Many cells lysed, as we would expect if Ca or Mg were playing a role in outer membrane rigidity (Fig. 3e). We placed the NC-7 on the microscope as quickly as possible after the addition of EDTA and acquired Movies S1 and S2. The movies indeed show the bursting of NC-7/34, suggesting loss of a containing structure
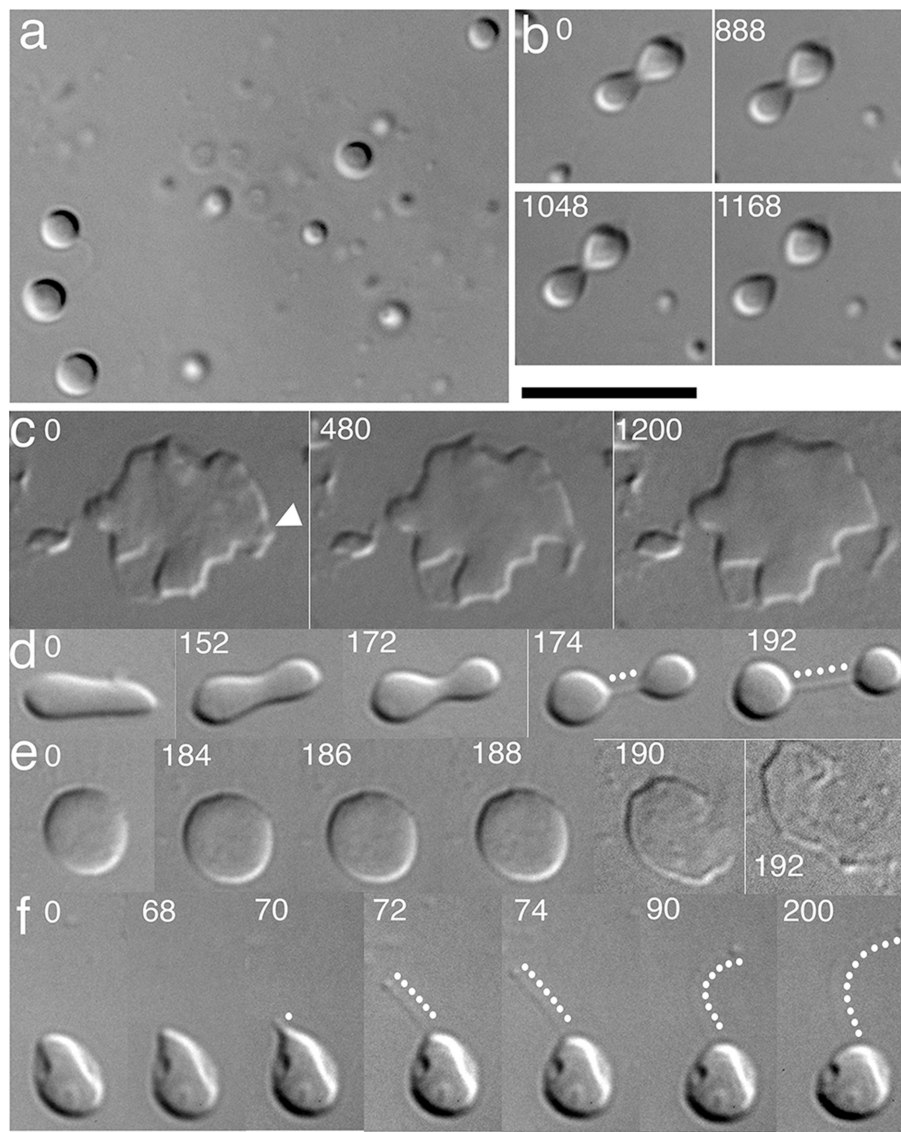
Fig. 3. Rounding, bursting, division and membrane extrusion of NC-7 following treatment with EDTA. (a) NC-7 cultured in MLB (340 mM NaCl) became spherical after treatment with 10 mM EDTA for 30 min. (b, c) Cell division of NC-7 in MLB (without EDTA) occurs by two mechanisms: (b) a cell elongates and constricts at the centre, or (c) a pleomorphic cell pinches off a bud (arrowhead). (d) EDTA induced rapid division of elongated NC-7/34 cells growing in low-osmolarity LB (34 mM NaCl). (e) EDTA induced lysis of some spherical NC-7/34 cells. (f) EDTA induced extrusion of membrane tubes from some NC-7/51 cells growing in low-osmolarity 51 mM NaCl LB. The numbers are time in seconds. White dots are placed along the membrane tubes connecting divided cells (d) or extruding from a cell (f). Bar, 10 µm.

when Ca and Mg were removed from LB. The bursting lysis suggests that the L form cells in low-osmolarity medium maintain some internal turgor pressure that is apparently contained by the outer membrane.

An interesting discovery was that treatment with EDTA induced rapid division of NC-7/34 cells that were already elongated. In the absence of EDTA, NC-7 cells can divide in two ways. One is to elongate, constrict in the middle and separate in two (Fig. 3b, Movie S3). Another is to bud from the mother cells (Fig. 3c, Movie S4). The frequency of both divisions was low under these normal growth conditions, and difficult to observe under the microscope regardless of

the shape of the cells. In contrast, when EDTA was added to NC-7/34 growing in low-osmolarity LB, cells that had elongated shapes such as cylinders or ellipsoids almost always divided within minutes (Fig. 3d, Movies S1, S2 and S5). This greatly enhanced frequency of division may be a response to reduced rigidity of the outer membrane upon removal of Ca/Mg. Symmetrical division was also more frequent for NC-7/34 in low-osmolarity LB than for NC-7 in MLB. The NC-7/34 cells typically remained connected by a thin tube after division (Fig. 3d).

EDTA also induced extrusion of membrane tubes, which originated from a small protrusion on cells that were slightly

deformed spheres (Fig. 3f, Movie S6). Sometimes the extrusions broke off and floated away from the cells (Movie S2: the cell labelled as releasing OMV). The membrane tubes apparently have some force causing the extrusions, perhaps from turgor pressure in the periplasm. They have a small refractile spot at the tip, which may have distorted the membrane and initiated the tubular extrusion.

FtsZ is apparently not involved in division of NC-7

We have previously shown that FtsZ-YFP (with its natural membrane tether FtsA) and FtsZ-YFP-mts (where mts is an amphipathic helix that targets FtsZ directly to the membrane) can form Z rings in liposomes and generate a constriction force [13, 17]. Therefore, we attempted to see if they can form Z rings and generate constriction force in L forms. First we expressed in NC-7 fully functional FtsZ with monomeric Venus YFP inserted between residues 55 and 56 of FtsZ [14] (Fig. S4a). FtsZ accumulations that might be related to Z rings were only observed in a few NC-7 cells (~3 %). Cells that had a constriction site rarely showed co-localized FtsZ, suggesting that FtsZ is not involved in NC-7 division. Second, we expressed FtsZ-YFP-mts, and found that this did not affect the growth and morphology of NC-7. In contrast, when FtsZ-YFP-mts was expressed in wild-type *E. coli* it inhibited division (data not shown). FtsZ-YFP-mts could form ring-like structures and spirals in some NC-7 cells, but they did not accumulate at the constriction site (Fig. S5a).

Gram-positive L forms can adapt to medium–low osmolality

A similar strategy was applied to the Gram-positive *B. subtilis*, which does not have an outer membrane. Strain LR-2 was established by Mercier *et al*. [3] as a genetically defined strain that can readily produce an L form. We generated an L form as previously described by Mercier *et al*. with slight modifications. The basic culture medium we used for selection and growth of the L form was one previously used for generating protoplasts, 2xPAB/SMM. This medium contains 0.5 M sucrose as osmoticum, and is 1246 mOsm kg^{-1} (Table 1). As with NC-7, our *B. subtilis* L form LR-2L grew at the bottom of the tube without shaking, but cell morphologies were different. Almost all LR-2L cells were spherical in 2xPAB/SMM (Fig. 4a), similar to NC-7 with its outer membrane compromised by CHIR-090 or EDTA.

To select for LR-2L adapted to low osmolality, the sucrose concentration was reduced in 2xPAB/SMM. When the LR-2L was cultured in 2xPAB/SMM containing 0.2 M sucrose, it could grow at essentially the same rate, but it reached a higher OD$_{600}$ (0.51) than that cultured in normal 2xPAB/SMM (OD of 0.34) (Fig. 4b). This suggests that 791 mOsm kg^{-1} is better than 1246 mOsm kg^{-1} for growth of this L form. The enhanced growth in 0.2 M sucrose was confirmed by the Coomassie assay of cell mass (Fig. S2b). LR-2L even survived in 2xPAB/SMM without sucrose (548 mOsm kg^{-1}), although the maximum OD only reached 0.07 (Fig. 4c) and the Coomassie assay confirmed minimal growth (Fig. S2b). Finally, we attempted to culture LR-2L in LB that contained

170 mM NaCl. This did not work. However, when 10 mM MgCl$_2$ was added to the LB, LR-2L could grow, suggesting that the divalent cation aided growth (Figs 4d and S2b). The maximum OD$_{600}$ for LR-2L in LB + 10 mM MgCl$_2$ was 0.055, which corresponds to about one-tenth of that in the 2xPAB/SMM containing 0.2 M sucrose.

Because Gram-positive bacteria are densely coated with negatively charged lipoteichoic acid, divalent cations may be involved in the resistance against the turgor pressure by linking and hardening lipoteichoic acid. To remove divalent cations from the culture medium, LR-2L growing at the bottom of a tube was diluted 50 times with the same medium in which MgCl$_2$ was replaced with 10 mM EDTA + 15 mM NaCl (adjusting the osmolality). The cells did not lyse and they retained their spherical morphology (Fig. 4e, f). This is different from NC-7, which lysed when the outer membrane was disrupted by the addition of EDTA. These results suggest a simple conclusion: that LR-2L can lower its cytoplasm to 440 mOsm kg^{-1} (the osmolality of LB + 10 mM MgCl$_2$) to adjust to the environment.

Interestingly, unlike NC-7, LR-2L could grow after abrupt transfer from 2xPAB/SMM (0.5 M sucrose) to 2xPAB/SMM (0 M sucrose) or to LB + 10 mM MgCl$_2$, skipping stepwise adaptations. This is very different from the slow adaptation of NC-7, where it requires stepwise dilution and took days to accumulate mutations. LR-2L apparently has some mechanism for a rapid adjustment to the hypoosmotic environment (see Discussion).

DISCUSSION

Our results show that the *E. coli* L form NC-7 could adapt to survive in very low-osmolality medium (132 mOsm kg^{-1}), and this ability is dependent on its outer membrane. The angular cell shape of the cells is apparently maintained by the rigidity of the outer membrane, because it is lost when the outer membrane is compromised by Chir-090 or EDTA. The cell lysis upon EDTA treatment suggests that the outer membrane is supporting some level of turgor. This suggests that the outer membrane may contribute to the mechanical rigidity of the cell wall of Gram-negative bacteria, the peptidoglycan layer of which is much thinner than that of Gram-positive bacteria. This suggestion is strongly supported by a recent study of Rojas *et al*. [18] showing that the outer membrane made a significant contribution to the rigidity of the Gram-negative cell wall. In that study Rojas *et al*. showed first that the outer membrane provided a major restraint on compression of the cell wall. They then showed that treatments that weakened the outer membrane compromised the ability of cells to survive oscillating osmotic shocks, and severely reduced the ability to obtain viable protoplasts or L forms. These latter experiments suggest that the outer membrane also provides resistance to expansion, and therefore contributes to the containment of turgor when the wall is subject to stress.

The contributions of the peptidoglycan and outer membrane to wall strength are not equal. Beta-lactam antibiotics lyse
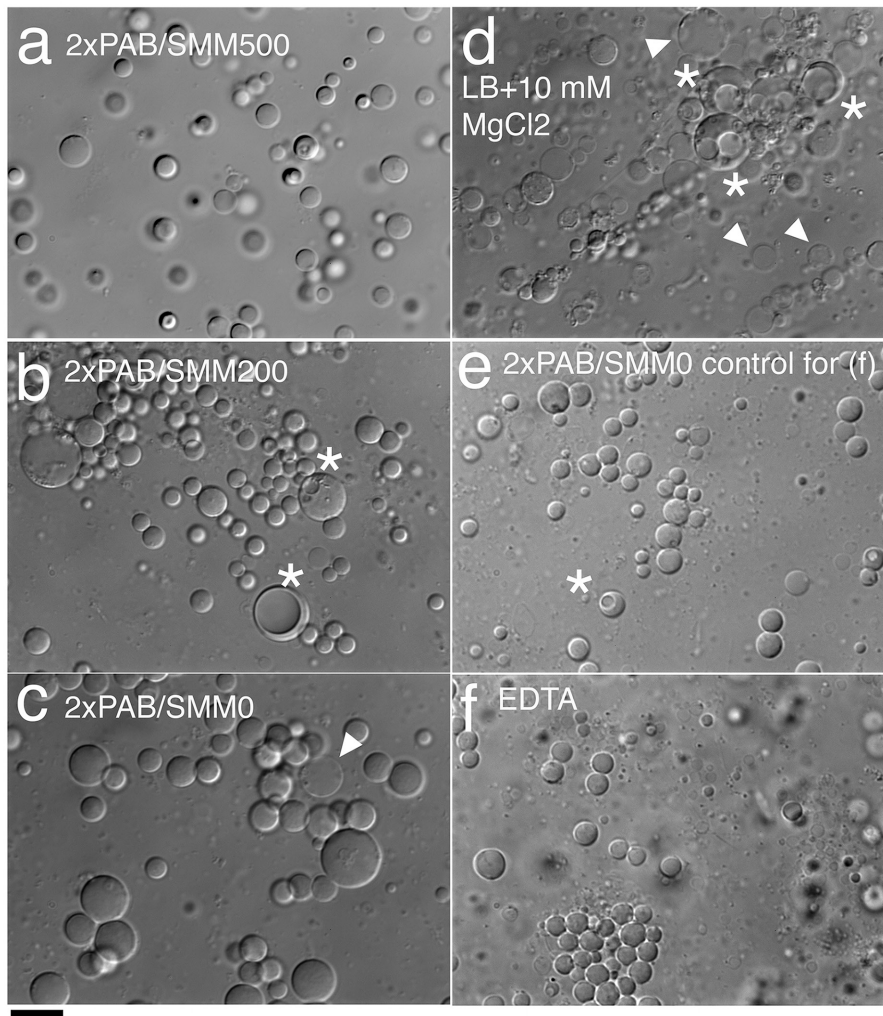
Fig. 4. Morphology of LR-2L growing at high and low osmolality. LR-2L grew in 2xPAB/SMM with 500 mM sucrose (a); 2xPAB/SMM with 200 mM sucrose (b); sucrose-free 2xPAB/SMM (c); or LB with 170 mM NaCl + 10 mM MgCl$_2$ (d). Arrowheads indicate the ghost membrane of dead cells. Asterisks show vacuole-like structures in the cytoplasm. (e) Control experiment for (f): LR-2L was diluted in sucrose-free 2xPAB/SMM, the same medium in which they were cultured. (f) LR-2L cultured in sucrose-free 2xPAB/SMM was diluted 50 times in the same medium with 10 mM EDTA, removing the divalent cation. Panel (e) should be the same as (c); the apparent difference in cell size may be due to uncontrolled culture conditions. Bars, 10 μm.

E. coli in LB, suggesting that the outer membrane itself cannot support the full turgor pressure. In contrast, disturbance of the outer membrane with EDTA in normal medium does not lyse *E. coli*. Therefore, the peptidoglycan contributes more than the outer membrane to supporting turgor pressure. Although the outer membrane of NC-7 can maintain the angular shape and prevent EDTA-mediated lysis in low-osmolality media, it probably cannot support a large turgor pressure. This suggests that when NC-7 adapts to low osmolality in our experiments, it lowers its cytoplasmic osmolality.

Although thin-section EM previously suggested that there is no outer membrane on NC-7 [8], we could actually observe by light microscopy (DIC, phase contrast and fluorescence) an apparent outer membrane blebbing from the spherical cells and separated by a large periplasmic space from an inner

membrane. Supporting the existence of an outer membrane, we also found that CHIR-090, which inhibits one of the LPS synthases involved in outer membrane synthesis, stopped NC-7 growth. We confirmed the previous report that NC-7 not only stopped growing but also rounded their cell shape when divalent cations were removed [9]. We conclude that the outer membrane has the rigidity to maintain a pleomorphic cell shape and to contain turgor pressure, and this rigidity requires divalent cations.

We do not know the cytoplasmic osmolality of NC-7 cultured in each different osmolality medium. In the case of normal *E. coli*, the turgor pressures were estimated as 3.1, 1.5 and 0.7 atm for external media of 30, 100 and 280 mOsm kg^{-1}, respectively [19]. Therefore, cytoplasmic osmolality in each case was 150, 160 and 308 mOsm kg^{-1}, respectively. This

indicates that *E. coli* can adjust their osmolality to external environments. If we assume that the same adjustment occurs in NC-7 (ignoring mutations and adjustments specific to the L form), NC-7/34 growing in 137 mOsm kg^{-1} medium would have a cytoplasmic osmolality of 170–200 mOsm kg^{-1} and a turgor pressure between 0.7 and 1.5 atm. If this turgor pressure is actually maintained, it is apparently supported by the outer membrane. However, the adaptation may have resulted in further lowering the cytoplasmic osmolality and turgor pressure.

As previously reported for the *B. subtilis* L form [2], NC-7 can divide symmetrically forming two spherical daughter cells, or it can form an extrusion that resolves to create a large and a small daughter cell. FtsZ is clearly not involved in division of the *B. subtilis* L forms [2]. Our results expressing FtsZ-YFP and FtsZ-YFP-mts suggest that FtsZ is not involved in division of NC-7.

Removing divalent cations from the culture medium by EDTA not only caused rounding of NC-7 cells, but also induced rapid division of cells that had an elongated shape. We suggest that the division may be driven primarily by excess inner/outer membrane. EDTA may cause an increase in the area of the outer membrane. Related examples are the shedding of vesicles induced by EDTA [20], and our own observation of release of vesicles (Movie S2) and projection of thin tubes (Fig. 3f, Movie S6).

Another mechanism that may contribute to division is the depletion force, where turgor pressure is lower in a spherical shape than in a cylindrical shape. This has been suggested by an *in vitro* observation where two spherical liposomes were electrically fused and subsequently divided into two spheres [21]. This division only occurred when PEG or dextran, macromolecules capable of generating a depletion force, were incorporated in the vesicles. A similar depletion force might be involved in L form division.

Finally we would like to speculate on a third mechanism by which reduced surface rigidity might induce division. If the rigidity of the outer membrane is preferentially reduced at the poles of cylindrical cells, perhaps because of their higher curvature, the two poles could swell and the middle of the cylinder reduce in diameter. Therefore, cytoplasm may be moved to the poles and form two spherical cells. This might provide a general mechanism by which cells with turgor pressure could divide without a constriction machinary. Cells might be able to induce division by changing the surface rigidity, although the division mechanism itself may still depend on excess membrane production and/or depletion force. This type of mechanism might be secondarily involved in cell divisions and/or some types of cell division where constriction machines such as a contractile ring are not present.

We also tested the L form LR-2L from *B. subtilis* and found that it could survive and grow at 548 mOsm kg^{-1}. There are two major differences from the results with *E. coli* NC-7. First, the minimum osmolality for LR-2L survival is much higher than that for NC-7. Second, LR-2L could survive direct dilution from 1246 to 548 mOsm kg^{-1}, and prolonged incubation did not generate mutants that could survive lower osmolality. This failure to adapt may be due to the genomic stability of LR-2L, which contrasts with the instability of NC-7 that permitted hundreds of mutations during adaptation. How does LR-2L survive a direct dilution into the lower osmolarity medium? One possibility is that it has a membrane reservoir that permits the cell to swell in volume, diluting its cytoplasmic osmolality. Another possibility would be a mechanism to rapidly secrete osmolytes, again diluting the cytoplasmic osmolality.

A recent study by Kawai *et al.* [22] showed that a *B. subtilis* L form can be generated directly in macrophages and survive in DMEM + 5 % serum, which is the culture medium for eukaryotic cells. This is interesting for our work because the osmolality of DMEM + 5 % serum is less than 350 mOsm kg^{-1}. Our LR-2L cells could not grow in medium of less than 440–548 mOsm kg^{-1}, and specifically did not grow when diluted into DMEM + 5 % serum (data not shown). There is, however, an earlier report that a *B. subtilis* L form can adapt to 350 mOsm kg^{-1} medium after long-term continuous culture [23]. However, the L form of Kawai *et al.* was created directly in the macrophage, presumably at ~350 mOsm kg^{-1}. That L form apparently survived when released from the macrophage into the cytoplasm or medium. Although Kawai *et al.* did not show whether this L form could divide and proliferate after release in DMEM + 5 % serum, this is presumably the path by which pathogenic L forms arise in the environments of humans and animals. The rigid outer-membrane of Gram-negative bacteria might equip them even better to generate L forms in macrophages.

References
1. **Klieneberger-Nobel E**. Origin, development and significance of L-forms in bacterial cultures. *J Gen Microbiol* 1949;3:434–443.
2. **Leaver M, Domínguez-Cuevas P, Coxhead JM, Daniel RA, Errington J**. Life without a wall or division machine in *Bacillus subtilis*. *Nature* 2009;457:849–853.
3. **Mercier R, Kawai Y, Errington J**. Excess membrane synthesis drives a primitive mode of cell proliferation. *Cell* 2013;152:997–1007.
4. **Mercier R, Kawai Y, Errington J**. General principles for the formation and proliferation of a wall-free (L-form) state in bacteria. *Elife* 2014;3 [Epub ahead of print 30 Oct 2014].
5. **Kawai Y, Mercier R, Wu LJ, Domínguez-Cuevas P, Oshima T et al.** Cell growth of wall-free L-form bacteria is limited by oxidative damage. *Curr Biol* 2015;25:1613–1618.
6. **Hofschneider PH, Martin HH**. Diversity of surface layers in L-forms of *Proteus mirabilis*. *J Gen Microbiol* 1968;51:23–33.
7. **Kroll HP, Gmeiner J, Martin HH**. Membranes of the protoplast L-form of *Proteus mirabilis*. *Arch Microbiol* 1980;127:223–229.
8. **Onoda T, Oshima A, Nakano S, Matsuno A**. Morphology, growth and reversion in a stable L-form of *Escherichia coli* K12. *J Gen Microbiol* 1987;133:527–534.

9. Onoda T, Enokizono J, Kaya H, Oshima A, Freestone P *et al*. Effects of calcium and calcium chelators on growth and morphology of *Escherichia coli* L-form NC-7. *J Bacteriol* 2000;182:1419–1422.

10. Rojas E, Theriot JA, Huang KC. Response of *Escherichia coli* growth rate to osmotic shock. *Proc Natl Acad Sci USA* 2014;111:7807–7812.

11. Pilizota T, Shaevitz JW. Origins of *Escherichia coli* growth rate and cell shape changes at high external osmolality. *Biophys J* 2014;107:1962–1969.

12. Kuhn A, Kellenberger E. Productive phage infection in *Escherichia coli* with reduced internal levels of the major cations. *J Bacteriol* 1985;163:906–912.

13. Osawa M, Anderson DE, Erickson HP. Reconstitution of contractile FtsZ rings in liposomes. *Science* 2008;320:792–794.

14. Moore DA, Whatley ZN, Joshi CP, Osawa M, Erickson HP. Probing for binding regions of the FtsZ protein surface through site-directed insertions: discovery of fully functional FtsZ-Fluorescent proteins. *J Bacteriol* 2017;199.

15. McClerren AL, Endsley S, Bowman JL, Andersen NH, Guan Z *et al*. A slow, tight-binding inhibitor of the zinc-dependent deacetylase LpxC of lipid A biosynthesis with antibiotic activity comparable to Ciprofloxacin. *Biochemistry* 2005;44:16574–16583.

16. Nikaido H. 2009. The limitations of LB medium. The MicroBlog ASM. http://schaechter.asmblog.org/schaechter/2009/11/the-limitations-of-lb-medium.html

17. Osawa M, Erickson HP. Liposome division by a simple bacterial division machinery. *Proc Natl Acad Sci USA* 2013;110:11000–11004.

18. Rojas ER, Billings G, Odermatt PD, Auer GK, Zhu L *et al*. The outer membrane is an essential load-bearing element in Gram-negative bacteria. *Nature* 2018;559:617–621.

19. Cayley DS, Guttman HJ, Record MT, Jr. Biophysical characterization of changes in amounts and activity of Escherichia coli cell and compartment water and turgor pressure in response to osmotic stress. *Biophys J* 2000;78:1748–1764.

20. Marvin HJ, ter Beest MB, Witholt B. Release of outer membrane fragments from wild-type *Escherichia coli* and from several *E. coli* lipopolysaccharide mutants by EDTA and heat shock treatments. *J Bacteriol* 1989;171:5262–5267.

21. Terasawa H, Nishimura K, Suzuki H, Matsuura T, Yomo T. Coupling of the fusion and budding of giant phospholipid vesicles containing macromolecules. *Proc Natl Acad Sci USA* 2012;109:5942–5947.

22. Kawai Y, Mickiewicz K, Errington J. Lysozyme counteracts β-lactam antibiotics by promoting the emergence of L-form bacteria. *Cell* 2018;172:1038–1049.

23. Gilpin RW, Patterson SK. Adaptation of a stable L-form of *Bacillus subtilis* to minimal salts medium without osmotic stabilizers. *J Bacteriol* 1976;125:845–849.

Edited by: J. Stülke and H. Strahl