Metabolite profiling and peptidoglycan analysis of transient cell wall-deficient bacteria in a new *Escherichia coli* model system

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Summary

Many bacteria are able to assume a transient cell wall-deficient (or L-form) state under favourable osmotic conditions. Cell wall stress such as exposure to β -lactam antibiotics can enforce the transition to and maintenance of this state. L-forms actively proliferate and can return to the walled state upon removal of the inducing agent. We have adopted *Escherichia coli* as a model system for the controlled transition to and reversion from the L-form state, and have studied these dynamics with genetics, cell biology and 'omics' technologies. As such, a transposon mutagenesis screen underscored the requirement for the Rcs phosphorelay and colanic acid synthesis, while proteomics show only little differences between rods and L-forms. In contrast, metabolome comparison reveals the high abundance of lysophospholipids and phospholipids with unsaturated or cyclopropanized fatty acids in *E. coli* L-forms. This increase of membrane lipids associated with increased membrane fluidity may facilitate

proliferation through bud formation. Visualization of the residual peptidoglycan with a fluorescently labelled peptidoglycan binding protein indicates *de novo* cell wall synthesis and a role for septal peptidoglycan synthesis during bud constriction. The DD-carboxypeptidases PBP5 and PBP6 are threefold and fourfold upregulated in L-forms, indicating a specific role for regulation of crosslinking during L-form proliferation.

Introduction

Bacteria possess a multitude of mechanisms to survive under harmful conditions. A well-known example is the formation of endospores as a response to nutrient depletion. Endospores are able to resist extreme environmental stress but can germinate when favourable conditions are re-established (Errington, 2003). Persister cells also remain viable in spite of adverse long-term antibiotic exposure (Lewis, 2010). A metabolic shutdown makes them insensitive to antibiotics. The discovery of persistence dates back over 60 years, but yet only recently, their clinical significance is being readdressed, specifically in chronic infections. First insights in the underlying molecular mechanisms have now become available (Kester and Fortune, 2014). L-forms are another antibiotic-tolerant phenotype that is known for many decades, but still remains cryptic, especially at the molecular level. Bacterial L-forms result from the transition of normal, walled bacteria to a cell wall-deficient state. The loss of an intact cell wall can be induced in several ways, e.g. exposure to cell wall-degrading compounds such as lysozyme, to inhibitors of cell wall-synthesis such as β -lactam antibiotics, but also through nutrient depletion (Allan *et al.*, 2009; Mearls *et al.*, 2012). As a consequence, L-forms are often resistant to antibiotics that are active on the cell wall (Domingue, 1982). Importantly, L-forms are viable and multiply by alternative reproduction mechanisms such as external and internal budding, extrusion-resolution and (a)symmetrical fission (Briers *et al.*, 2012a). L-forms are subdivided according to the extent of cell wall-deficiency and their stability. Whereas spheroplasts have some residual peptidoglycan, protoplasts completely lack any

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cell wall. Unstable L-forms can revert to the walled state when they are no longer exposed to the inducer, whereas stable L-forms continuously reproduce as such even in the absence of such an inducer (Allan *et al.*, 2009).

The clinical significance of L-forms is controversial and subject of much debate (reviewed by Domingue and Woody, 1997; Mattman, 2001; Allan *et al.*, 2009). Whereas some attribute virulent properties to L-forms, the hypothesis that unstable L-forms may be an escape route in response to cell wall stress has more support. Recurrent transitions and reversions between walled and unwalled cellular states may contribute to relapsing bacterial infections. In this view, virulence and pathogenicity is only associated with the walled state, whereas the cell wall-deficient state is responsible for prolonged persistence in the human body. Other authors dispute any clinical role for L-forms (Onwuamaegbu *et al.*, 2005; Lantos *et al.*, 2014; Schnell *et al.*, 2014). They emphasize the lack of uniformity of techniques used to identify L-forms and argue that current evidence for their clinical significance is mainly based on case reports. Generally, all contributors agree that more studies are needed to clarify the clinical significance of L-forms (Allan *et al.*, 2009). However, research on cell wall-deficiency is challenging because of the fastidious character of L-forms and their pleomorphic nature. There has been a lack of genetically tractable L-form model systems that can be produced under standardized and reproducible conditions. Recently, important progress was made with the introduction of a *Bacillus subtilis* (Leaver *et al.*, 2009; Domínguez-Cuevas *et al.*, 2012; Mercier *et al.*, 2012) and a *Listeria monocytogenes* (Dell'Era *et al.*, 2009; Briers *et al.*, 2012b) L-form model system. However, both models are stable L-forms that do not revert when the inducer is removed (except by genetic restoration of cell wall synthesis; Kawai *et al.*, 2014). Ranjit and Young (2013) described lysozyme-induced *E. coli* spheroplasts that spontaneously recover a rod shape; however, it has not been reported whether these spheroplasts can replicate autonomously in the presence of the inducing factor.

We present here a reversible model of transient cell wall-deficiency based on *E. coli* MG1655, in which all cells quantitatively undergo transition and reversion. Therefore, this model allows to study transition, L-form reproduction and reversion in a controlled manner within a single model. Using this model, we show that in spite of limited differences at the proteome level, the L-form metabolome is featured by a high abundance of (lyso)phospholipids that increase membrane fluidity. A residual amount of peptidoglycan precursors and peptidoglycan turnover products is detected in actively multiplying L-forms. We show the location of the low amount of newly synthesized

peptidoglycan during L-form reproduction and visualize the resumption of the full peptidoglycan synthetic capacity during reversion to rods.

Results

Visualization and quantification of transition, L-form growth and reversion

An ideal *in vitro* L-form model system should meet several requirements: (i) the transition and reversion should be quick, quantifiable and at will, (ii) to enable easy collection for analyses by demanding molecular techniques, the L-forms should be able to grow in liquid media and finally, (iii) their growth rate should not be too slow and they should grow as colonies on a plate. The L-form model system described here meets all these prerequisites and is based on *E. coli* L-forms described by Joseleau-Petit and colleagues (2007). *E. coli* MG1655 – one of the most tractable organisms for molecular research – is induced to the cell wall-deficient state in hypertonic M broth supplemented with 45 µg ml⁻¹ β-lactam cefsulodin (MCef45). In this liquid medium supplemented with cefsulodin, they are able to propagate in the absence of an organized cell wall and can easily revert to rods upon antibiotic removal (Fig. 1). The model describes thus unstable L-forms that can revert.

Transitions mostly occur at the septum or at the poles, and occasionally at other positions along the cell wall, and invariably result in dumping an empty sacculus (Video Clip S1). Successful transition results in actively reproducing L-forms in 25–50% of the cases, whereas failed transitions result in prompt lysis. The transition rate has been quantified every 30 min after addition of cefsulodin. Transitions do not take place synchronously. Instead, most L-forms escape from the sacculus between 60 and 300 min after cefsulodin exposure. A transient subpopulation of transition intermediates that remain attached to the sacculus, increases and decreases again as the balance between the rate of initial protoplast escape and the formation of completely formed L-forms shifts (Fig. 2A).

Once transition is completed, the suspension only contains L-forms that multiply infinitely in the absence of an intact cell wall, as long as cefsulodin was present in the medium. The reproduction trajectory differs depending on the culture conditions. In a liquid, shaking culture with cefsulodin, L-forms multiply through the formation of relatively small buds that are split off, possibly because of shear forces. On an agar pad with cefsulodin, initial doublings of L-form cells often appear to be rather symmetrical (binary fission-like), whereas later divisions become more asymmetrical with multiple, simultaneous membrane blebbing events occurring in a single L-form.

Transition

Growth

Reversion

Fig. 1. Model system of transient cell wall-deficiency. Exemplary time-lapse series are shown for the transition from rods to L-forms (upper), multiplication of L-forms (middle) and the reversion from L-forms to rods (lower). Time points (min) and scale bars (7.5 μm) are indicated. Transitions mostly take place at the septum but also along the lateral wall or at the poles. Spherical cell wall-deficient cells then multiply by symmetrical and asymmetrical budding, and the formation of protrusions. Upon cefsulodin removal, cells revert to a more rod-like shape (white arrow heads), they elongate and eventually get normal dimensions.

As a result, many pleiomorphic cells are formed. Tubular protrusions that are eventually split off can also be observed (Video Clip S2). Growth rate in the mid-exponential phase of L-forms (MCef45 broth) is lower ($\mu = 0.16 \text{ h}^{-1}$) than the growth rate of rods in hypertonic medium without cefsulodin (M broth) ($\mu = 0.48 \text{ h}^{-1}$) or in reference lysogeny broth (LB) broth ($\mu = 0.56 \text{ h}^{-1}$). In addition, the maximal density of L-forms in the stationary phase is about one third lower than the density of rods in the same hypertonic medium. Continuous, spontaneous lysis of a fraction of the cells during proliferation will certainly contribute to a reduced maximal density that can be achieved.

In liquid culture, reversion starts with filamentous outgrowths from the L-form surface, which are eventually pinched off. On an agar pad, pleiomorphic cells deform to a more elongated shape, followed by outwards, filamentous growth at ectopic poles, leading to branched cells (Video Clip S3). Already 30 min after cefsulodin removal, approximately 50% of the population shows such intermediate cell shapes, indicating a quick reversion, and the proportion of L-forms gradually drops in the next 4.5 h. An almost isomorphic population of rods appears after 5 h (Fig. 2B).

Altogether, this model system of *E. coli* MG1655 L-forms induced with cefsulodin provides a good framework to study transition, L-form reproduction and reversion *in vitro*.

Transposon mutagenesis underscores the necessity of the Rcs phosphorelay and colanic acid synthesis for L-form survival

A total of 5760 mutants from a random transposon library were screened by replica plating on both M and MCef45 agar for the capacity to undergo transition and multiply as L-form cells. Fourteen transposon mutants in nine different genes could be identified as growing on M agar but not on MCef45 agar (Table 1). Seven mutants are defective in the colanic acid biosynthesis cluster (*wzc*, *wcaD*, *wcaE*, *wcaK* and *wcaL*), two others in the synthesis of colanic acid precursors (*galE*, *ugd*) and five more in the Rcs two-component system (*rscC*, *rscD*). The latter system is activated by cell wall stress and regulates up to 150 genes, including genes involved in colanic acid synthesis. All mutants could be confirmed by plating the corresponding single-gene, in-frame deletion mutants from the KEIO collection (Baba *et al.*, 2006).

Fig. 2. Transition and reversion rate. The (A) transition rate and (B) reversion rate of cefsulodin-induced *E. coli* L-forms have been quantified in liquid culture. Subpopulation of rods (black), L-forms (white) and intermediate forms (grey) have been counted. Intermediate forms during transition (exposure to cefsulodin) are cells that did not complete transition entirely and appear as protoplasts attached to a remaining sacculus (A). During reversion (removal of cefsulodin), intermediate forms represent these cells that started reversion by the formation of long protrusions, but do not have a rod shape yet (B). Time starts after addition (A) or removal (B) of cefsulodin and quantification takes place every 30 min up to 300 min. In every condition, between 50 and 300 cells were classified. Each bar represents the mean of three independent experiments.

We used an MG1655 strain equipped with a chromosomal *rprA-lacZ* fusion to confirm Rcs activation in L-forms (Majdalani *et al.*, 2002). The strain was grown either as regular rods (in LB or hypertonic M broth) or as L-forms (in MCef45 broth), and β -galactosidase activity was measured with o-nitrophenyl- β -D-galactoside. In hypertonic M broth, a twofold upregulation takes place, followed by a further 75-fold increase in MCef45. These data clearly confirm the activation of the Rcs phosphorelay in L-forms (Fig. S1).

Two other mutants were resistant to $45 \mu\text{g ml}^{-1}$ cefsulodin because they grew as rods on MCef45 agar plates (which can be easily differentiated from L-form colonies due to a less mucoid appearance). These mutants have a transposon insertion in *hrpB*, encoding a DNA helicase. This gene is located directly upstream of *mrcB* that codes for PBP1B, the target of cefsulodin. Upregulation of transcription of *mrcB* by an internal promoter of the transposon has most likely outperformed the effect of cefsulodin. Consistently, the in-frame *hrpB* deletion mutant from the KEIO collection (Baba *et al.*, 2006), which misses this internal promoter, grows as normal L-form colonies on MCef45 agar plates, excluding any essential role of HrpB for L-form growth. In conclusion, (activation of) colanic acid synthesis is of crucial importance for L-form growth.

The L-form proteome is highly similar to the proteome of normal rods

A comparison of the proteome of *E. coli* MG1655 rods grown in LB or M broth, and L-forms in MCef45 broth was performed through two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and mass spectrometric analysis. The proteome of L-forms surprisingly appears to be more identical to the proteome of rods grown in LB (92.1%) than in M broth (86.8%) ($n = 419$). There were no unique or strongly overabundant protein spots present in

Table 1. Transposon mutagenesis and identification of genes essential for L-form growth.

Gene	Hits	Accession N°	Function	Group
1 wcaD	2	EG13572	Colanic acid polymerase	Biosynthesis
2 wcaE	1	EG13573	Putative colanic acid biosynthesis glycosyl transferase	Biosynthesis
3 wcaK	2	EG13577	Putative galactokinase, colanic acid biosynthesis protein, pyruvyl transferase	Biosynthesis
4 wcaL	1	EG12652	Putative colanic acid biosynthesis glycosyl transferase	Biosynthesis
5 wzc	1	EG13568	Protein-tyrosine kinase, involved in translocation of the growing chain	Biosynthesis
6 ugd	1	EG13407	UDP-glucose 6-hydrogenase (converts UDP-D-glucose in UDP-D-glucuronate)	Precursor synthesis
7 galE	1	EG10362	UDP-galactose-4-epimerase (converts UPD-D-galactose to UDP-D-glucose or opposite)	Precursor synthesis
8 rcsC	2	EG10822	Hybrid sensory kinase in two-component regulatory system with RcsB	Sensor
9 rcsD	3	EG12385	Phosphotransfer intermediate protein in two-component regulatory system with RcsBC	Sensor

Fourteen confirmed transposon mutants are distributed over nine different genes. The number of hits per gene is indicated. Their corresponding accession number and function is given. The different genes can be subdivided in genes involved in colanic acid biosynthesis, synthesis of colanic acid precursors and the Rcs two-component sensor system that activates colanic acid synthesis.

Fig. 3. Overview proteome comparison between rods and L-forms. A majority of spots ($n = 355$ or 84.7% of the panproteome) is present under three conditions tested. The proteome of L-forms does not reveal unique spots ($n = 0$ or 0%) and shares more spots with the proteome of cells grown in reference LB medium ($n = 386$ or 92.1%) than with the proteome of cells grown under the same hypertonic conditions in absence of cefsulodin ($n = 356$ or 84.9%).

the L-form samples. In contrast, 32 spots present either in LB, M or both conditions were absent or less abundant in L-forms (Fig. 3). These proteins belong to diverse functional categories (Table S1). Two isoforms of isocitrate lyase are absent under hypertonic conditions with and without cefsulodin, whereas a third isoform is present but strongly less abundant ($< 10\%$) under these conditions. Isocitrate lyase catalyses the first step in the glyoxylate shunt in the TCA cycle, suggesting that the glyoxylate shunt is less active under hypertonic conditions. The inhibitor of vertebrate lysozyme (Ivy) is only detected in M broth. It has been reported that the expression of Ivy is activated by the two-component Rcs system that is triggered by cell wall stress (Callewaert *et al.*, 2009). Although the Rcs system is also strongly activated in L-forms (Fig. S1), Ivy appears to be absent in the latter. Possibly periplasmic Ivy may be lost in the absence of an intact cell wall. Two isoforms of the outer membrane protein A (OmpA) are detected in L-forms. Their presence indicates that L-forms should have at least a rudimentary outer membrane, although the exact nature and structure of the outer membrane of L-forms remains unknown. Altogether, changes in the proteome of L-forms were found to be only limited.

L-forms have an increased number of (lyso)phospholipids with unsaturated or cyclopropanized fatty acid chains

The large differences in morphology, lifestyle and reproduction mechanism of L-form argue for a different metabolism in spite of the largely conserved proteome. The metabolome is the downstream outcome of the transcriptome and proteome and is therefore closely

associated with the physiology of a certain phenotype of the cell (Putri *et al.*, 2013). To obtain an accurate snapshot of the actual physiological differences between rods and L-forms, we compared the metabolome of rods grown in both reference broth (LB) and hypertonic broth (M), and of L-forms grown in hypertonic broth with cefsulodin (MCef45). Rods grown in LB and exposed to cefsulodin lysed quickly, making sampling impossible. Among three conditions tested, 310 different ions were assigned to 467 putative metabolites (including mass isomers) from a total of 884 molecules contained in a genome scale metabolic model of *E. coli* (Table S2) (Feist *et al.*, 2007; Fuhrer *et al.*, 2011). A wide variety of metabolite classes were covered: carbohydrates, lipids, nucleotides, amino acids, vitamins, cofactors, energy metabolism, terpenoids, polyketides and glycans. About half of the detected metabolites ($n = 154$) had a significantly different concentration under at least one of the conditions $\{\log_2[\text{met}(\text{condition X})/\text{met}(\text{condition Y})] > 0.5; P < 0.01\}$ (Table S2). The differences in metabolite concentration were used to perform hierarchical clustering. Both biological and technical replicates of each condition were grouped together, confirming the reproducibility of each metabolic profile (Fig. S2). A principal component analysis allowed the identification of the most prominent metabolites that explain the majority of variance between the different conditions (Fig. S3). The first two components explain 94.6% of total variance and were retained for analysis. The first component representing 85.3% of the variance separates the metabolic profile of L-forms (MCef45) from those of rods (LB and M) and has high component loadings for (cyclopropanized) phospholipids and lysophospholipids. The second component (9.3%) mainly distinguishes the samples from rods grown in hypertonic broth from the other conditions and is characterized by a high component loading for reduced glutathione (Table S3). These results show that the three conditions are mainly differentiated by their differences in (lyso)phospholipid metabolism and the antioxidant glutathione.

In absolute terms, there is a clear increase of the total abundance of phospholipids in L-forms (+65%/+46%, compared with LB and M respectively) with cyclopropanized fatty acids (+107%/+62%) and unsaturated acyl chains (+39%/+35%) responsible for the most prominent changes, whereas the number of phospholipids with saturated fatty acids are only slightly increased (+18%/+1%). Specifically, four out of 14 detected phospholipids have a significantly increased concentration in L-forms ($\log_2 > 0.5; P < 0.01$), specifically phosphatidyl ethanolamine (16:1) ($\log_2$ -fold increases of +0.42/+0.74) and its cyclopropanized form (+1.14/+0.81), phosphatidyl ethanolamine (18:1) (+0.94/+0.58) and cyclopropane phosphatidylglycerol (16:0) (+0.96/+0.52) (Fig. 4). Peak

Fig. 4. Volcano plots of (lyso)phospholipids. A pairwise comparison of the abundance of 14 detected phospholipids (circles) and 11 detected lysophospholipids (triangles) between L-forms (MCef45) and rods (LB) is given (see also Table S2). The $\log_2$ of the fold changes are plotted (x-axis) versus the $-\log_{10}$ of the corresponding P -value (y-axis). Most extremely different metabolites of both groups are annotated.

heights of the 10 other, generally less abundant phospholipids are less different among the three conditions (Table S2). Lysophospholipids or mono-acyl phospholipids show even more drastic changes in the L-form metabolome (+385%/+531%, compared with LB and M respectively). Especially lysophospholipids with an ethanolamine headgroup and longer acyl chains (18:1; 16:1 and 16:0; +3.05/+3.16, +4.62/+4.38 and +4.18/+4.23 $\log_2$ -fold changes, respectively) have a higher concentration in L-forms. In contrast, lysophospholipids with a glycerol headgroup and shorter acyl chains (12:0 and 14:1) are underrepresented in L-forms (−1.13/−0.26 and −2.09/−0.91 respectively) (Fig. 4). In conclusion, not only the total number of (lyso)phospholipids is significantly higher in L-forms, but also the relative proportion of phospholipids with unsaturated and cyclopropanized fatty acid chains has increased.

The decreased level of reduced glutathione in L-forms compared with rods grown in LB and M (−2.26 and −4.09 respectively) may indicate an increased level of oxidative stress in the cell wall state. Reduced glutathione is one of the strongest bacterial antioxidants and constitutes 99.5% of the total cellular amount of glutathione under unstressed conditions (Smirnova and Oktyabrsky, 2005). Oxidative stress would result in an increase of oxidized glutathione at the expense of the reduced form. However, oxidized glutathione was not detected. An alternative

explanation for the decreased level of reduced glutathione could be that the total pool of glutathione is reduced in L-forms. Slightly decreased levels of glutathione have been previously reported in slow-growing bacteria, and also the L-forms analysed here have a slower growth rate ($\mu = 0.16 \text{ h}^{-1}$ versus 0.48 and 0.56 h^{-1} for rods grown in hypertonic and LB broth respectively). This is possibly linked to the lower amount of glutamate (−1.86/−1.10), which is required for glutathione synthesis (Tweeddale *et al.*, 1998).

The residual amount of newly synthesized peptidoglycan collocates with buds and constriction sites during L-form reproduction

Different metabolites related to peptidoglycan synthesis [N-acetyl-muramine acid, UDP-N-acetylmuramoyl-L-alanine, UDP-N-acetyl-glucosamine, meso-diaminopimelic acid (m-DAP)] and to peptidoglycan turnover and recycling [(N-acetyl-D-glucosamine)-1, 6-anhydrous-N-acetylmuramyl-tetrapeptide, (N-acetyl-D-glucosamine)-1,6-anhydrous-N-acetylmuramyl-tripeptide, L-alanine-D-glutamate-m-DAP, L-alanine-D-glutamate-m-DAP-D-alanine] are detected in L-forms (Table S2). Although mostly in a lower abundance compared with rods, their presence suggests active peptidoglycan synthesis and degradation. This is consistent with the finding

Fig. 5. Peptidoglycan labelling with recombinant PBD-KZ-GFP. Rods undergoing transition (left), growing L-forms (middle) and reverting L-forms (right) were fixated and permeabilized prior to incubation with PBD-KZ-GFP. Unbound fluorescent protein was removed. The phase contrast and fluorescence channels are shown. Arrow heads indicate colocalization of peptidoglycan and the bud constriction site during L-form growth. Intensities of the middle panel have been increased for clarity. Scale bar is 2 μ m.

that residual peptidoglycan in cefsulodin-induced L-forms accounts for 7% of the normal amount in rods and that its synthesis is essential for growth and probably required for cell division (Joseleau-Petit *et al.*, 2007). To locate peptidoglycan (residues) during transition, growth and reversion, we used a fusion protein that combines a peptidoglycan-binding domain (PBD) derived from the endolysin of bacteriophage ϕ KZ and GFP (PBD-KZ-GFP) (Briers *et al.*, 2007). Similar as observed for rods, addition of purified PBD-KZ-GFP did not bind to the L-forms, indicating the presence of an effectively shielding outer membrane in L-forms as in rods. Only after permeabilization of the outer membrane, the peptidoglycan layer of rods is accessible for the fluorescent peptidoglycan-binding protein (Briers *et al.*, 2007). We used chemical fixation and permeabilization to remove the outer membrane barrier. Fixation is needed to stabilize the osmotically unstable L-forms, preventing lysis during permeabilization of the outer and inner membrane of the cells. After fixation and permeabilization of the cells, we labelled cells with purified PBD-KZ-GFP at different stages between the rod- and cell wall-deficient state (Fig. 5).

During transition, the cell wall breaks open at the septum or the pole and the cytoplasm is released as a protoplast, apparently completely devoid of peptidoglycan

remnants. A relatively intact sacculus is left behind. In contrast, growing and multiplying L-forms show clear local accumulations of peptidoglycan, colocalizing with protruding buds. The peptidoglycan does not sharply delineate the cell border as observed in rods, but is more blurred. In general, the fluorescence intensity appears to increase with the bud size but remains lower than that of an intact sacculus in rods. During constriction of the buds, peptidoglycan mostly locates at the constriction site. When cefsulodin is removed and reversion starts, a strong fluorescence surrounds the protrusions, often expanding over the spherical surface of the L-forms, or along the deformed, elongated shape of the reverting L-form.

L-forms have an increased level of DD-carboxypeptidases PBP5 and PBP6

The complete loss of the sacculus during transition and the apparent absence of any peptidoglycan suggest *de novo* peptidoglycan synthesis in L-forms. Penicillin-binding proteins (PBPs) are required for the final steps of peptidoglycan synthesis, specifically for polymerization of the precursors (transglycosylation) and cross-linking of adjacent chains (transpeptidation). To quantify changes

Table 2. PBP profile during the L-form transition-growth-reversion cycle.

	Hypertonic medium	Transition	L-forms	Reverted L-forms
PBP1A	10	0,5	3	10
PBP1B	10	0	0	10
PBP1C	10	4	10	10
PBP2	10	10	10	10
PBP3	10	5	10	10
PBP4	10	20	10	10
PBP5	10	10	30	10
PBP6	10	12	40	10
PBP7	10	10	10	10

The profile of PBPs was compared for rods grown in hypertonic medium, rods undergoing transition to the L-form state, multiplying L-forms and L-forms reverting to rods. The fluorescence intensities were measured and normalized for comparison.

in the PBP profiles during conversion from the rod to the cell wall-deficient state and back, we used Bocillin FL (Molecular Probes), a fluorescent penicillin, as a labelling reagent for PBPs (Table 2) (Zhao *et al.*, 1999). A reference PBP profile of *E. coli* MG1655 rods has been used for identification of the different identified PBPs (Fig. S5). Most prominent changes in the PBP profile were a three-fold and fourfold upregulation of PBP5 and PBP6 in L-forms in comparison with rods respectively. PBP5 and PBP6 are two out of four DD-carboxypeptidases encoded by *E. coli*, cleaving the terminal D-alanine from the pentapeptide. Therefore, one would expect more terminal D-Ala residues to be removed from peptidoglycan. This would in turn reduce the degree of cross-linking by transpeptidases PBP1A and PBP1B (Templin *et al.*, 1999; Heseck *et al.*, 2004), influencing the cell shape. Previously, it has been reported that elevated levels of PBP5 result in osmotically stable spherical *E. coli* cells (Markiewicz *et al.*, 1982). The lack of terminal D-Ala residues may also explain why approximately a doubling of DAP-DAP cross-links were detected in *E. coli* L-forms at the expense of DAP-D-Ala cross-links (Joseleau-Petit *et al.*, 2007). Interestingly, lysozyme-induced *E. coli* spheroplasts lacking PBP5 or PBP6 show a delayed and altered reversion to the rod shape (Ranjit and Young, 2013), further suggesting a modulating role for these enzymes and the degree of cross-linking in the cell wall-deficient state.

Bifunctional transglycosylase/transpeptidases PBP1A and PBP1B are detected at lower levels in samples from cells undergoing transition and L-forms. However, this observation could be biased significantly by the acylation of these PBPs by the L-form inducing agent cefsulodin, converting those PBPs to an unsuitable binding target of Bocillin FL. Indeed, it has previously been shown that the transglycosylase moiety of PBP1B is necessary for L-form

growth, making the absence of PBP1B in L-forms unlikely (Joseleau-Petit *et al.*, 2007). Extensive washing of the samples did not change this outcome, indicating an extremely low deacylation rate of the PBP-cefsulodin complex.

Discussion

Bacteria encounter many adverse conditions in their natural life. It was quickly understood that L-forms may be a possible escape route upon exposure to cell wall-inhibiting antibiotics, immune serum, complement and bacteriophages, and return to the walled state when the inducing factor is removed (Dienes and Weinberger, 1951). Decades of research on the phenomenon of cell wall-deficiency and survival as L-forms followed and yielded a multitude of induction protocols and descriptions of their morphology, reproduction mechanisms and cytological properties. However, knowledge of the molecular cell biology of (transient) cell wall-deficiency is still in its infancy due to two main reasons: first, the research field peaked before the introduction of modern molecular tools in microbiology, and second, of the lack of tractable model systems to study transition, growth and reversion. Expanding from *E. coli* L-forms described by Joseleau-Petit and colleagues (2007), we here describe a model of transient cell wall-deficiency. The three steps in a cycle of transient cell wall-deficiency (transition, L-form reproduction and reversion) can be fully controlled and visualized. This allows to study transition, L-form reproduction and reversion both at the population level (using omics technologies) and at the single cell level (using fluorescence microscopy). An important feature is the quantifiable nature of the model. Induced L-forms and reverting L-forms grow on a plate and can be quantified as single colonies that grow from a single cell, which is in contrast to most other L-form systems that do not grow on agar plates. This allows to study quantitative effects of different growth conditions or mutants.

Transition takes place at the septum and recently formed cell poles but only rarely along the lateral cell wall, which is mostly the case in *B. subtilis* L-forms (Domínguez-Cuevas *et al.*, 2012). Cefsulodin inhibits the transpeptidase activity of the bifunctional penicillin-binding proteins PBP1A and PBP1B, the major enzymes for peptidoglycan synthesis in *E. coli*. PBP1B is located both at the septum, poles and lateral cell wall (Bertsche *et al.*, 2006), but it may not be surprising that a cell wall defect – large enough for protoplast escape – most easily occurs at the septal site.

Whereas division follows a relatively uniform path in *E. coli* rods, division in L-forms ranges from binary fission-like division, asymmetrical fission to the formation of small buds. Especially on agar pads, aberrant and extremely

pleomorphic cells can be formed. This is consistent with our previous hypothesis that L-forms have a proliferative mechanism that is typically less efficient, less coordinated and slower (Briers *et al.*, 2012a). Although the L-form genome encodes all elements for a functional divisome and elongasome, the structural organization and tight links between both machineries are lost. In this perspective, it has been found previously that MreB becomes dispensable, while FtsZ remains essential for viability of cefsulodin-induced *E. coli* L-forms (Joseleau-Petit *et al.*, 2007).

All observed genetic requirements necessary for L-form growth and multiplication can be attributed to the formation of the capsular polysaccharide colanic acid. The Rcs two-component sensor kinase system, genes involved in precursor synthesis and synthesis of the colanic acid polymer were identified. The presence of colanic acid was already obvious from the mucoid appearance of *E. coli* L-form colonies, and its requirement was confirmed previously for cefsulodin- and penicillin-induced *E. coli* L-forms (Joseleau-Petit *et al.*, 2007; Glover *et al.*, 2009). Considering the finite number of transposon mutants, the existence of other essential genes for L-form growth cannot be excluded. However, because of the randomness of the library (approximately 1% of the mutants are confirmed to be auxotroph, corresponding to the theoretical proportion) and multiple independent hits in the same genes, it is unlikely that other large clusters of related genes could be identified.

Especially in view of the drastic changes of the phenotype of L-forms, the proteome of L-forms shows remarkably high similarity to the wild-type proteome. No single additional or significantly upregulated proteins were identified in the 2D-map of the L-form proteome. However, when the metabolome was compared, large changes were observed with half of the detected ions having a significantly different concentration ($154/310$; $\log_2 > 0.5$; $P < 0.01$). This indicates that most of the changes take place at the level of the metabolism through differences in enzyme activity, in allosteric regulation of enzymes and in fluxes through metabolic pathways.

There is an obvious shift of C-atoms from carbohydrates, amino acids and nucleotides to (lyso)phospholipids. The higher total amount of phospholipids in L-forms may be surprising because the transition from rod to sphere is accompanied by a decrease in the surface/volume ratio, which would suggest a lower need for phospholipids. However, Bendezú and de Boer (2008) have previously shown that spherical *E. coli mreB* mutants are unable to adjust their phospholipid synthesis rate to changes in the surface area, resulting in a membrane excess. The increased proportion of phospholipids observed in *E. coli* L-forms also further supports the hypothesis that L-form reproduction generally depends on

biophysical membrane dynamics driven by the imbalance between cell membrane and volume (Leaver *et al.*, 2009; Briers *et al.*, 2012a).

Membrane fluidity is a key factor that allows membrane deformations to effectively result in new progeny. In *B. subtilis* L-forms, sufficient membrane fluidity is achieved through the synthesis of branched chain fatty acids (Mercier *et al.*, 2013). *E. coli* produces only straight-chain fatty acids but likely achieves the same outcome with an increased level of phospholipids having unsaturated and cyclopropanized acyl chains (Fig. 4, Table S2). Indeed, both unsaturated and cyclopropanized fatty acids have a poor acyl chain packing capacity in the phospholipid bilayer (Perly *et al.*, 1985). Cyclopropanized phospholipids result from the addition of a methylene group to the double bond of an unsaturated fatty acid. This conversion occurs as a conditional, post-synthetic modification when bacteria enter the stationary phase, but also during the adaptation of bacteria in response to drastic changes in the environment (Chang and Cronan, 1999). Although the exact physiological role of cyclopropanized phospholipids remains unclear, it has been suggested that they improve the chemical stability of the membrane, e.g. against oxidative and osmotic stress, without altering the physical properties of the membrane (Grogan and Cronan, 1997; Asakura *et al.*, 2012). In the same perspective, the drastically increased abundance of lysophospholipids in L-forms may lead to a higher membrane fluidity as well. Lysophospholipids result from the Lands cycle to remodel the composition of the fatty acids of the phospholipids by cycles of deacylation and reacylation. The Lands cycle is important for the maturation of the cytoplasmic membrane and adaptation to the environment (Shindou *et al.*, 2009). Alternatively, lysophospholipids may be an intermediate product of phospholipid catabolism. This process starts with β -oxidation, but the secondary degradation products are not present in such an elevated amount in the L-form metabolome. Therefore, a hyperactive Lands cycle is the most plausible explanation for the abundant lysophospholipids. This indicates a very active turnover to adapt membrane fluidity.

Although a protoplast escaping from the sacculus is apparently completely devoid of peptidoglycan, we have shown here that new peptidoglycan is synthesized during L-form growth and multiplication. This peptidoglycan colocalizes and coincides with the formation of buds, although it is less delineated and more diffuse. Mostly the whole bud is covered, but in many occasions only the region around the constriction site between mother and daughter cell is labelled, suggesting that septal peptidoglycan synthesis is involved in bud constriction. This is consistent with the finding that cefsulodin-induced *E. coli* L-forms are inhibited by piperacillin that blocks

PBP3, necessary for septal peptidoglycan synthesis (Joseleau-Petit *et al.*, 2007). A functional, asymmetric Z-ring might still induce constriction assisted by septal peptidoglycan synthesis to pinch off the newly formed bud. From this perspective, the reproduction mechanism of this *E. coli* L-form model system is more sophisticated at the molecular level compared with the *B. subtilis* model system (Leaver *et al.*, 2009), which has a FtsZ-independent division mechanism.

Reversion of cefsulodin-induced L-forms to rods on an agar pad follows a remarkable similar path as lysozyme-induced spheroplasts via a series of aberrant cells, which are often filamentous, branched and thickened (Ranjit and Young, 2013). Reversion in a shaking, liquid culture is more straightforward with filamentous protrusions that are pinched off eventually. These protrusions are covered by an apparently organized layer of peptidoglycan, which may even expand over the cell surface of the mother cell. This shows that removal of the inducer quickly results in the activation of a functional peptidoglycan synthesis machinery to produce mature peptidoglycan. The PBP profile of completely reverted cells also becomes identical to rod-shaped cells. As such, the peptidoglycan synthesis machinery appears to be a flexible system that produces *de novo* peptidoglycan after protoplast escape, can sustain growth as a L-form spheroplast and produce a complete new sacculus around protrusions.

There is an ongoing debate about the definition of L-forms. Although Joseleau-Petit and colleagues (2007) originally named the cells analysed in this study L-form-like cells, this was immediately refuted by Young (2007) who considered them as L-forms. He proposed to classify L-forms as class I (unstable L-forms that can revert) and class II (stable L-forms that cannot revert). This suggestion was further generalized by Allan and colleagues (2009) who introduced a unifying definition for L-forms, which he differentiated into four types: unstable and stable spheroplast L-forms and unstable and stable protoplast L-forms. Where spheroplasts still possess some remaining cell wall structure, protoplasts are completely devoid of any cell wall. According to this definition, this work studied unstable spheroplast L-forms.

In conclusion, we report here an *E. coli* model system to study the L-form transition–reversion cycle on a molecular basis using state-of-the-art techniques. A better understanding of transient cell wall-deficiency *in vitro* will provide firmer ground to analyse and interpret this phenomenon *in vivo*. Besides a contribution to the debate on the relation between transient cell wall-deficiency and disease, this model also offers unique properties to study *de novo* cell wall synthesis, cell wall stress response and may offer a glimpse on the reproduction of more primitive bacterial life forms (Briers *et al.*, 2012a; Errington, 2013).

Experimental procedures

Bacterial strains, media and growth conditions

All experiments were performed with *E. coli* K-12 MG1655 (Bachmann, 1996), except the in-frame, single-gene knockout strains of the KEIO collection, which have an *E. coli* K-12 BW25113 background (Baba *et al.*, 2006). The tested mutants from the KEIO collection were $\Delta wzc::Km^R$, $\Delta wcaD::Km^R$, $\Delta wcaE::Km^R$, $\Delta wcaK::Km^R$, $\Delta wcaL::Km^R$, $\Delta ugd::Km^R$, $\Delta galE::Km^R$, $\Delta rcsD::Km^R$, $\Delta rcsC::Km^R$ and $\Delta hrpB::Km^R$. MG1655 $\Delta(argF-lac)U169::rprA142-lacZ$ (kindly provided by Sarah Ades, Department of Biochemistry and Molecular Biology, The Pennsylvania State University, University Park, PA; Laubacher and Ades, 2008) was used to measure Rcs phosphorelay activation. β -galactosidase activity was measured as described previously (Zhang and Bremer, 1995). For routine growth or as reference medium, LB broth was used. L-forms were grown in hypertonic medium (M broth) [3 g l⁻¹ beef extract (Becton, Dickinson and Company), 10 g l⁻¹ bacteriological peptone (LabM), 5 g l⁻¹ yeast extract (LabM), 5 g l⁻¹ NaCl (Acros Organics), 0.01 M MgSO₄ (Acros Organics), 0.23 M sucrose (Acros Organics)], supplemented with 45 μ g ml⁻¹ cefsulodin (Sigma-Aldrich). To induce L-forms, cells grown overnight in M broth were used as inoculum. Agar plates contained 1.2 (w/v) % bacteriological agar No. 1 (Lab M). For growth of the KEIO knockout strains, 50 μ g ml⁻¹ kanamycin was added to the medium. All growth was performed at 30°C.

Microscopy

The (fluorescent) microscopic images were acquired using two different microscopes. Images from Fig. 1 were obtained using a Leica TCS SPE confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany), operated by the LEICA LAS AF interface. Sample incubation temperature was controlled at 30°C, using an incubation chamber permanently attached to the microscope ('The Cube', Life Imaging Services, Basel, Switzerland). An HCX PL FLUOTAR 100 $\times$ /1.30 oil-immersion objective was used. Transmission light images were obtained using phase contrast. All other images were acquired with a temperature-controlled (Okolab Ottaviano, Italy) Ti-Eclipse inverted microscope (Nikon, Champigny-sur-Marne, France) equipped with a TI-CT-E motorized condensor, a GFP filter (Ex 472/30, DM 495, Em 520/35) and a CoolSnap HQ2 FireWire CCD-camera. Operation of the microscope was done using NIS-Elements (Nikon). Cells (2 μ l) were dropped on agar pads in a shallow depression of a microscope slide and covered by a semi-attached cover slip. The agar pad comprised either LB, hypertonic or hypertonic + cefsulodin medium with 1.2 (w/v) % agar. Processing of the images was performed with the same LEICA LAS AF software, NIS ELEMENTS VIEWER, open source software IMAGEJ (<http://rsbweb.nih.gov/ij/>) and CORELDRAW X4 (Corel Corporation, Ottawa, Canada) was used for final image assembly and contrast/brightness adjustments.

Transposon mutagenesis

A transposon knockout library of *E. coli* MG1655 was constructed using λ NK1324, which carries a mini-Tn10

transposon with a chloramphenicol resistance gene, according to the protocol described by Kleckner and colleagues (1991). To confirm random insertion, the library was screened for auxotrophy on M9 minimal medium with 0.5% glucose. Eight out of 600 random clones (1.33%) did not grow, corresponding to the theoretical frequency of 1%. Overnight grown mutants in LB were replica-plated with a 96-well pin replicator on M and MCef45 agar plates. The plates were then incubated overnight at 30°C. Mutants that were able to grow on M agar but not in the L-form state on MCef45 agar plates were after a double confirmation selected for determination of the transposon inserting site using the method described by Kwon and Rieke (2000).

2D-PAGE and mass spectrometry

2D-PAGE in gel-trypsinization of selected spots and mass spectrometric analysis was performed as described by Lecoutere and colleagues (2012). Briefly, all separations in two dimensions were carried out using GE Healthcare devices and reagents and according to the manufacturer's instructions. Isoelectric focusing (IEF) was performed using immobilized pH gradient (IPG) strips (24 cm Immobiline DryStrips with linear pH gradient range 4–7). IEF was run in an Ettan IPGphorII. Subsequently, the second dimension was run in 1 mm thick vertical gels (15% polyacrylamide) using an Ettan DALTsix. Protein spots were visualized by colloidal Coomassie brilliant blue G-250 overnight staining. Image acquisition was performed using a calibrated flatbed ImageScanner, combined with LABSCAN software. 2-DE maps were analysed and spot data generated using IMAGEMASTER 2D PLATINUM software. Selected Coomassie blue spots were excised using wide-bore tips and destained. The proteins were reduced and alkylated, whereafter the gel slices were sequentially hydrated, dehydrated and dried. Trypsin Gold (Promega, Madison, WI, USA; final concentration of 12.5 µg ml⁻¹) was added, followed by overnight digestion. Finally, peptides were extracted from the gel by sonication. Prior to mass spectrometric analysis, peptide samples were dried in a vacuum centrifuge and desalted using ZipTip C₁₈ pipette tips (Millipore, Bedford, MA, USA). Peptides were separated by liquid chromatography (LC) with a linear 5–60 (v/v) % acetonitrile gradient and subsequently identified by ESI-MS/MS (LCQ Classic, ThermoFinnigan) in an *m/z* range of 300–1500. All MS data were analysed using SEQUEST v.1.2 within PROTEOME DISCOVERER v.1.2 (ThermoFinnigan) and MASCOT v. 2.4 (Matrix Sciences) against the *E. coli* MG1655 genome (NC_000913.2; 4408 protein entries). Results from both search engines were evaluated using SCAFFOLD v.3.6 at a minimal peptide and protein probability threshold of 95% and 99% respectively.

Metabolite profiling and analysis

Bacteria grown to mid-exponential growth phase ($OD_{600nm} = 0.6$; 0.45 and 0.35 for cells grown in LB, M and MCef45 respectively) were sampled for metabolite profiling by fast filtration as previously described (Link *et al.*, 2013). Briefly, a culture volume corresponding to a biomass of 2 ml culture at $OD_{600nm} = 1.0$ was vacuum-filtered through a 0.45 µm pore size nitrocellulose filter (Millipore, Billerica, MA, USA) and

washed with 2 ml ammoniumcarbonate solution (75 mM, pH 7.0). The filters were incubated in 3 ml ethanol (60%) at 78°C for 2 min, and the samples were snap-frozen in liquid nitrogen to be stored at –80°C until further processing. Metabolite extracts were dried under vacuum at 30°C and re-suspended in 100 µl water. Metabolites were profiled using negative mode flow injection-time-of-flight mass spectrometry (Agilent 6520), and detected ions were annotated based on accurate mass measurements using the strategy previously reported (Fuhrer *et al.*, 2011). In brief, the mass of detected anions was compared with the list of calculated masses of reference metabolites compiled from the genome-scale metabolic model of *E. coli* (Feist *et al.*, 2007) after manual curation. A mass tolerance of 1 mDa was allowed, and only the best hit within this tolerance was accepted. Furthermore, analysis of frequent mass shifts was performed to eliminate annotations based on neutral losses and ion adducts. Metabolite levels were relatively quantified by integration of the ion intensity signal, which was previously shown to be a good approximation for the metabolite concentration (0.1–50 µM tested) in a complex biological matrix (Fuhrer *et al.*, 2011). Statistical analysis and principal component analysis was performed using MATLAB R2010b (Mathworks, Natick, MA, United States). Good symmetry of the volcano plots of pairwise comparisons (*t*-test) of all detected ions confirmed that there are no relevant differences in the sampled biomass (Fig. S4).

Fixation, permeabilization and staining with PBD-KZ-GFP

L-forms in the mid-logarithmic phase ($OD_{600nm} = 0.35$), cells undergoing transition or reversion were fixated during 1.5 h at room temperature in freshly prepared 4 (v/v) % formaldehyde (Sigma-Aldrich) either dissolved in hypertonic broth without (reverting cells) or with 45 µg ml⁻¹ cefsulodin (cells in transition or growing L-forms). After fixation, cells were washed with the corresponding medium. Subsequently, cells were permeabilized for 30 min with a mixture of 1 (v/v) % Triton X-100 (Acros Organics), 50 mM EDTA (Acros Organics) and 0.1% SDS (Sigma-Aldrich) dissolved in PBS. Cells were washed and incubated for 15 min using an excess of PBD-KZ-GFP. PBD-KZ-GFP was produced and purified as described in Briers and colleagues (2007). Unbound PBD-KZ-GFP was removed by washing. Samples were visualized as described above.

Determination of PBP profile

Rods grown in M broth and L-forms grown in MCef45 broth were harvested in the stationary phase ($OD_{600nm} = \sim 0.9$ and ~ 0.6 respectively). The transition and reversion samples had about a 1:1 rods/L-forms ratio. Their optical densities corresponded to ~ 0.6 and ~ 0.9 respectively. Cells were spun down at 16 000 g for 1 min, and membrane extracts were prepared thereof. Thirty micrograms of membrane protein in 15 µl phosphate buffer (50 mM pH 7.0) was labelled at 37°C for 30 min with a final concentration of 5 µM of Bocillin FL and separated on a 7% acrylamide, 3.3% cross-linkage gel SDS-PAGE. When appropriate, samples were incubated at 37°C with clavulanic acid at a final concentration of 10 µg ml⁻¹ or

EDTA at a final concentration of 10 mM for 30 min before labelling, so as to avoid degradation of the fluorescent penicillin by β -lactamases. The PBP3s were visualized directly on the gel by fluorescence using Typhon9410 (Amersham Biosciences) with an excitation wavelength of 588 nm and emission filter 520BP40. These assays were repeated threefold.

Acknowledgements

The authors greatly acknowledge the technical assistance by Tuo Zheng, Sander Govers and Erik Royackers. Y. B. held a postdoctoral mandate of the KU Leuven Research Fund.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Induction of the Rcs pathway. Activation of the Rcs two-component system is measured as β -galactosidase activity in a *rprA-lacZ* reporter strain. β -galactosidase activity is expressed in Miller units. The strain was grown in LB, M (both rods) and MCef45 (L-forms). Averages and standard deviations of three replicates are shown.

Fig. S2. Hierarchical cluster analysis. Hierarchical clustering (pearson correlation and euclidean distance) was performed based on the abundance of ions detected in metabolic samples of rods grown in LB and M broth and L-forms grown in MCef45 broth. Technical (2) and biological replicates (2–3) were included separately in the analysis. Ions with a higher and lower abundance are shown in red and blue respectively. This plot demonstrates that technical and biological replicates are more similar to each other than samples of different growth conditions, which serves as a quality check for the sample preparation and the measurement procedure.

Fig. S3. Principal component analysis. All detected components among the different technical and biological replicates of the different conditions were used for a principal component analysis. The first and second principal components were plotted. The first principal component is depicted on the x-axis and contains 85.3% of the variation, while the second principal component on the y-axis comprises 9.3% of the variation.

Fig. S4. Volcano plots. Pairwise comparisons of all detected components of the metabolome under different conditions confirm that all samples have approximately the same biomass.

Fig. S5. Bocillin FL binding profile of PBPs from *E. coli* envelopes. Cell envelopes were prepared and labelled, and the PBPs detected and quantified as described in *Experimental procedures*. The specific bands corresponding to PBPs are indicated in the second column. The third column is the quantified value for each individual PBPs $\pm$ standard deviation for three independent analyses. It is important to note that each PBP has a different specific binding affinity for Bocillin FL. The measured intensities thus do not reflect the relative abundance of the different PBPs in the cell, but are a function of both amount and specific binding affinity.

Table S1. Mass spectrometric identification of selected protein spots. Spots unique to one of the conditions or spots showing strongly different abundance in at least one of the conditions were isolated, trypsinized and identified by mass spectrometry. Their abundance is expressed as intensity of the spot (%int) or as volumetric fraction (%vol), taking into account the area of the spot. Number of spectra and coverage of the protein sequence are given for each spot.

Table S2. All detected and annotated metabolites.

Table S3. Loadings of first two principal components.

Video Clip S1. Transition. Upon addition of cefsulodin ($45 \mu\text{g ml}^{-1}$) to a culture of *E. coli* MG1655 rods on an agar pad, the protoplasts escape at the septum, poles and along the lateral cell wall. About 25–50% of the transitions result in viable, actively reproducing L-forms; in all other cases, the released protoplasts eventually lyse.

Video Clip S2. L-form growth and multiplication. L-forms reproduction trajectories on an agar pad are either binary fission-like, asymmetric fission or via the formations of bulges, buds and protrusions. Failing division may result in giant pleomorphic cells that eventually lyse.

Video Clip S3. Reversion. Upon removal of cefsulodin and washing, a culture of *E. coli* L-forms on an agar pad starts reversion to rods. Initially, elongated, filamentous or branched intermediates are formed. After some divisions at irregular septum sites, rods obtain normal dimensions and undergo mid-cell division. Rods overgrow the remaining L-forms because of their higher growth rate.