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

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Abstract

Many bacteria are able to assume a transient cell wall-deficient (or L-form) state under favorable 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 labeled 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 three- and four-fold 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 favorable conditions are reestablished (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 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-form, 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 more studies are needed to clarify the clinical significance of L-forms (Allan et al., 2009). However, research on cell wall-deficiency is challenging due to 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; Dominguez-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 Escherichia 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: (1) the transition and reversion should be quick, quantifiable and at will, (2) to enable easy collection for analyses by demanding molecular techniques, the L-forms should be able to grow in liquid media and finally, (3) 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 et al. (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 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 (Figure 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 (Movie 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 minutes after addition of cefsulodin. Transitions do not take place synchronously. Instead, most L-forms escape from the sacculus between 60 and 300 minutes 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 (Figure 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 due to 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. As a result, many pleiomorphic cells are formed. Tubular protrusions that are eventually split off can also be observed (Movie 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 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, pleomorphic cells deform to a more elongated shape, followed by outwards, filamentous growth at ectopic poles, leading to branched cells (Movie S3). Already 30 minutes 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 hours (Figure 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 5,760 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 (*rcsC, rcsD*). 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). 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 two-fold 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 (Figure S1).

Two other mutants were resistant to 45 µg/ml 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 hrbB, 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 hrbB 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 HrbB 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 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 the L-form samples. In contrast, 32 spots present either in LB, M or both conditions were absent or less abundant in L-forms (Figure 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 catalyzes the first step in the glyoxylate shunt in the TCA cycle, suggesting 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 (Figure 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 \textit{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 ($\log_2(\text{met}_X/\text{met}_Y) > 0.5; P<0.01$) (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 (Figure S2). A principal component analysis allowed the identification of the most prominent metabolites that explain the majority of variance between the different conditions (Figure 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 to 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 fourteen 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) (Figure 4). Peak heights of the ten 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 to 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) (Figure 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 to 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 analyzed here have a slower growth rate (μ = 0.16 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 to rods, their presence suggests active peptidoglycan synthesis and degradation. This is consistent with the finding 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 $\phi$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 labeled cells with purified PBD-KZ-GFP at different stages between the rod- and cell wall-deficient state (Figure 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, colocating 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 in the PBP profiles during conversion from the rod to the cell wall-deficient state and back, we used Bocillin FL, a fluorescent penicillin, as a labeling 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 (Figure S5). Most prominent changes in the PBP profile were a three- and four-fold upregulation of PBP5 and PBP6 in L-forms in comparison to 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; Hesek 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. First, because the research field peaked before the introduction of modern molecular tools in microbiology, and second, because of the lack of tractable model systems to study transition, growth and reversion. Expanding from *E. coli* L-forms described by Joseleau-Petit *et al.* (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 wildtype 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, since 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 (Briers *et al.*, 2012a; Leaver *et al.*, 2009; Mercier *et al.*, 2013).

Membrane fluidity is a key factor that allows membrane deformations to effectively result in new progeny. In *Bacillus 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 (Figure 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 Land’s 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 collocates 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 labeled, 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 to 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 et al. (2007) originally named the cells analyzed 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 et al. (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 analyze 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 a *E. coli* K-12 BW25113 background (Baba *et al.*, 2006). The tested mutants from the KEIO collection were Δ*wzc*: Km^R^, Δ*wcaD*: Km^R^, Δ*wcaE*: Km^R^, Δ*wcaK*: Km^R^, Δ*ugd*: Km^R^, Δ*galE*: Km^R^, Δ*rcsD*: Km^R^, Δ*rcsC*: Km^R^ and Δ*hrpB*: Km^R^. MG1655 Δ*(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) (3g/l beef extract (Becton, Dickson and Company), 10 g/l bacteriological peptone (LabM), 5 g/l yeast extract (LabM), 5 g/l NaCl (Acros Organics), 0.01 M MgSO_4^ (Acros Organics), 0.23 M sucrose (Acros Organics)), supplemented with 45 µg/ml 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 µg/ml 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 Figure 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 100x/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 motorised 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 µl) 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 λNK1324, which carries a mini-Tn10 transposon with a chloramphenicol resistance gene, according to the protocol described by Kleckner *et al.* (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 (1.13%) 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 Ricke (2000).

**2D-PAGE and mass spectrometry**

2D-PAGE, in gel-trypsinization of selected spots and mass spectrometric analysis was performed as described by Lecoutere et al. (2012). Briefly, all separations in two dimensions were carried out using GE Healthcare devices and reagents and according to the manufacturer’s instructions. Iso Electric Focusing (IEF) was performed using 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 analyzed 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; final concentration of 12.5 µg/ml) 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). Peptides were separated by LC with a linear 5-60 (v/v) % ACN gradient and subsequently identified by ESI-MS/MS (LCQ Classic,
ThermoFinnigan) in an m/z range of 300–1500. All MS data were analyzed 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; 4.408 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., 2012). Briefly, a culture volume corresponding to a biomass of 2 mL culture at OD600 = 1.0 was vacuum-filtered through a 0.45 μm pore size nitrocellulose filter (Millipore, Billerica, MA, USA) and washed with 2 mL ammonium carbonate solution (75 mM, pH 7.0). The filters were incubated in 3 mL ethanol (60%) at 78°C for two minutes 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 resuspended 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 to 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 to 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 (Figure 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 hours 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 cefsulodin (cells in transition or growing L-forms). After fixation, cells were washed with the corresponding medium. Subsequently, cells were permeabilized for 30 minutes 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 minutes using an excess of CWB-KZ-GFP. PBD-KZ-GFP was produced and purified as described in Briers et al. (2007). Unbound PBD-KZ-GFP was removed by washing. Samples were visualized as described above.

**Determination of PBP profile**

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Rods grown in M broth and L-forms grown in MCEF45 broth were harvested in the stationary phase (OD600nm=~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 16,000g 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 (Molecular Probes) 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 PBPs 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 three-fold.

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Figure 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 bar (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.
Figure 2: Transition and reversion rate

The transition rate (A) and reversion rate (B) of cefsulodin-induced *E. coli* L-forms has 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 don’t have a rod shape yet (B). Time starts after addition (A) or removal (B) of cefsulodin and quantification takes place every 30 minutes up to 300 minutes. In every condition, between 50 and 300 cells were classified. Each bar represents the mean of three independent experiments.
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%).
Figure 4: Volcano plots of (lyso)phospholipids

A pairwise comparison of the abundance of fourteen detected phospholipids (circles) and eleven 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.
Figure 5: Peptidoglycan labeling 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 channel are shown. Arrows indicate colocation 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 µm.
Table 1: Transposon mutagenesis and identification of genes essential for L-form growth.

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.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Hits</th>
<th>Accession N°</th>
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<td>2</td>
<td>EG13572</td>
<td>Colanic acid polymerase</td>
<td>Biosynthesis</td>
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<td>6</td>
<td>1</td>
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<tr>
<td>7</td>
<td>1</td>
<td>EG10362</td>
<td>UDP-galactose-4-epimerase (converts UPD-D-galactose to UDP-D-glucose or opposite)</td>
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<td>EG12385</td>
<td>Phosphotransfer intermediate protein in two-component regulatory system with RcsBC</td>
<td>Sensor</td>
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Table 2: PBP profile during the L-form transition-growth-reversion cycle

The profile of penicillin binding proteins (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.

<table>
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<tr>
<th>PBP</th>
<th>Hypertonic medium</th>
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<th>L-forms</th>
<th>Reverted L-forms</th>
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