The analysis of cell division and cell wall synthesis genes reveals mutationally inactivated *ftsQ* and *mraY* in a protoplast-type L-form of *Escherichia coli*

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**Abstract**

Cell division and cell wall synthesis are tightly linked cellular processes for bacterial growth. A protoplast-type L-form *Escherichia coli*, strain LW1655F⁺, indicated that bacteria can divide without assembling a cell wall. However, the molecular basis of its phenotype remained unknown. To establish a first phenotype–genotype correlation, we analyzed its *dcw* locus, and other genes involved in division of *E. coli*. The analysis revealed defective *ftsQ* and *mraY* genes, truncated by a nonsense and a frame-shift mutation, respectively. Missense mutations were determined in the *ftsA* and *ftsW* products yielding amino-acid replacements at conserved positions. FtsQ and MraY, obviously nonfunctional in the L-form, are essential for cell division and cell wall synthesis, respectively, in all bacteria with a peptidoglycan-based cell wall. LW1655F⁺ is able to survive their loss-of-functions. This points to compensatory mechanisms for cell division in the absence of murein sacculus formation. Hence, this L-form represents an interesting model to investigate the plasticity of cell division in *E. coli*, and to demonstrate how concepts fundamental for bacterial life can be bypassed.

**Introduction**

Cell division and cell wall synthesis are highly linked processes integrated into the metabolism for bacterial growth (Nanninga, 1998). In *Escherichia coli*, cell division takes place at the midcell after the chromosome is duplicated and segregated into two daughter nucleoids. After formation of a ring-like structure (Z-ring) composed of FtsZ, other cell division-specific Fts-proteins localize at the constriction site in a striking linear hierarchy (Goehring & Beckwith, 2005). There, they co-ordinate the invagination of the cytoplasmic membrane (CM), and the inward growth of the murein (peptidoglycan) sacculus, and the outer membrane (OM) layer. Although significant progress has been made in the identification of cell division proteins and their localization to their site of action, little is known about their true functional role, and the factors which are involved to co-ordinate specifically the growth of the cell wall during the late steps of cell division (Goehring & Beckwith, 2005).

Here, we examined a mutant derived from the *E. coli* K-12 laboratory strain (Fig. 1), which had irreversibly lost the ability to form a cell wall. The mutant had been isolated by long-term cultivation of its cell wall-bearing parental strain W1655F⁺ in the presence of penicillin and sucrose without any mutagenic treatments, until a protoplast-type L-form emerged (Schuhmann & Taubeneck, 1969). The mutant, designated LW1655F⁺, is able to grow surrounded only by the CM. Electron microscopical studies have shown spherical cells, lacking the typical components of the Gram-negative envelope, e.g. flagellae and fimbriae, the OM, the murein layer, and the periplasmic space (Schuhmann & Taubeneck, 1969; Gumpert et al., 1971). The lacking envelope components account for osmotic fragility, for resistance against cell wall-active antibiotics (e.g. ampicillin), for sensitivity towards inhibitors which cannot pass the OM, for resistance to coliphage infection, and for the inability of the L-form to mobilize genetic material via conjugation (Schuhmann & Taubeneck, 1969; Gumpert et al., 1971, 2002). Growth of the L-form is confined to complex media, indicating strict dependency on rich nutrient supply. Since its isolation 35 years ago, no reversion to the cell wall-bearing phenotype has been observed. Neither penicillin as selective agent nor osmotic stabilizers are required to maintain the mutant phenotype (Gumpert et al., 2002).
Considering the importance of the cell wall for essential cellular processes such as cell division, morphogenesis, or homeostasis (Nanninga, 1998), it is puzzling how *E. coli* is able to grow without assembling a proper cell wall. Despite previous reports indicating that LW1655F<sup>+</sup> may have bypassed the link of cell division and cell wall synthesis, the genetic basis of its phenotype was never investigated. We hypothesized that its stable phenotype is associated with changes in the genes responsible for cell division and for cell wall synthesis, compared with normal *E. coli*.

After confirming the identity of the L-form as an *E. coli* K-12 descendant, we performed a comparative sequence analysis of selected cell division and cell wall synthesis genes with the reference genes of *E. coli* K-12 MG1655F<sup>−</sup> (Blattner et al., 1997). The results obtained provide strong evidence that mutant LW1655F<sup>+</sup> offers a chance to investigate major deviations of bacterial division in the future.

### Materials and methods

#### Bacterial strains and growth conditions

*Escherichia coli* strains LW1655F<sup>+</sup> and W1655F<sup>+</sup> were cultivated in brain heart infusion (BHI) broth (Difco). Thirty milliliters BHI broth were inoculated with 3 mL (LW1655F<sup>+</sup>) and with 0.3 mL (K-12 or W1655F<sup>+</sup>) of overnight cultures and shaken on a rotary shaker (200 r.p.m., 37 °C). For plating, media were solidified with 1.2% (w/v) Bacto Agar (Difco) and supplemented with 10% (v/v) horse serum.

#### Nucleotide sequence determination and analysis

Genomic DNA was extracted with QiaTip columns according to the lysis and purification protocols recommended by the manufacturer (Qiagen, FRG) and used as the template to amplify polymerase chain reaction (PCR) products. Oligonucleotides of 17–25 nucleotides (nt) deduced from the *E. coli* K-12 MG1655 genome (Acc. no. U00096) served as primers. Taq and PFU polymerase (PeqLab, FRG) were used according to instructions of the supplier. Long-range PCR was performed with Platinum Taq High Fidelity DNA Polymerase-Mix according to the instruction of the supplier (Invitrogen, FRG). Primers were designed within the GAP software (Staden et al., 2000) and synthesized at MWG-Biotech (Ebersberg, FRG). After PCR product purification (Qiagen), sequencing was carried out with the dye terminator method on ABI 377 and ABI3700 sequencers, according to the instructions of the manufacturer (Applied Biosystems). The nucleotide sequence of both strands of the PCR products were determined in all cases. Sequence data were processed and edited with the GAP software version 4.6 (Staden et al., 2000). Nucleotide sequence and primary structure analysis was performed with BLAST (Altschul

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**Table 1.** Markers of the parent strain W1655F<sup>+</sup> determined in LW1655F<sup>+</sup>

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein variation</th>
<th>DNA changes in LW1655F&lt;sup&gt;+&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rRNA</td>
<td>99.93 % identity&lt;sup&gt;1&lt;/sup&gt;; A to C mismatch at pos. 80 is present in the L-form and its parent (reference is the rme of <em>Escherichia coli</em> K-12 MG1655F&lt;sup&gt;−&lt;/sup&gt;)</td>
<td></td>
</tr>
<tr>
<td>relA</td>
<td>Protein disruption</td>
<td>1337 bp insertion at position 261</td>
</tr>
<tr>
<td>spoT</td>
<td>84QD insertion</td>
<td>CAGGAT insertion at position 252</td>
</tr>
<tr>
<td>creC</td>
<td>Frame shift</td>
<td>G → C at position 230</td>
</tr>
<tr>
<td>metB</td>
<td>Frame shift</td>
<td>GC deletion at position 141–142</td>
</tr>
</tbody>
</table>

*Position counted from the start triplet of the respective gene.
1517 bp of the 16S rRNA gene of the L-form and parent W1655F<sup>+</sup> were amplified and compared with the rme.

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**Fig. 1.** Pedigree of *Escherichia coli* K12-derived strains and lineage of the protoplast-type L-form LW1655F<sup>+</sup>. AO, acridine orange for curing the fertility-plasmid F; UV, ultraviolet treatment; spont., spontaneous mutation; EM8-Lac, eosin-methyleneblue lactose selection; X-ray, treatment with γ-irradiation; λ<sup>+</sup>, λ<sup>−</sup>, absence, and presence of phage λ, respectively. The conditions of LW1655F<sup>+</sup> isolation (Schuhmann & Taubeneck, 1969) are indicated.
et al., 1990) and with ClustalW (www.clustalw.genome.ad.jp). All DNA sequences obtained in this study are deposited at the NCBI under the following accession nos.: AY616568 (the complete dcw from mraZ to lpxC), AY616658 (16S rRNA gene), AY616615 (creC), AY616617 (ftsK), AY616613 (ftsN), AY616614 (metB), AY616590 (metB), AY616590 (minC), AY616589 (minD), AY616598 (minE), bankit708954 (relA insertion), AY616610 (spoT), AY616619 (sulA), AY616600 (ftsB, ygbQ), AY616602 (zapA), AY616597 (zipA).

Oligonucleotides used for PCR amplification and sequencing

All the oligonucleotides to amplify PCR products and to sequence the dcw cluster, full-length genes, and the 16S rRNA gene of the protoplast-type L-form LW1655F⁺ by primer walking on genomic DNA (f, forward orientation; r, reverse orientation) are listed in the Supplemental Files document, Table S1, Table S2, and Table S3, respectively.

Results and discussion

Confirmation of the E. coli K-12 lineage

To confirm the identity of the mutant strain LW1655F⁺, we determined known genetic markers according to the pedigree of Escherichia coli K-12 laboratory strains (Bachmann, 1996) and performed a 16S rRNA gene analysis. We amplified DNA fragments by PCR from the L-form with oligonucleotides deduced from E. coli K-12 MG1655F⁺ genome (Blattner et al., 1997), and then determined their nucleotide sequences. The results are summarized in Table 1, which show that LW1655F⁺, as outlined in Fig. 1, is an E. coli K-12 derivative. This facilitated the screening and comparative analysis of selected L-form genes with the reference genes of E. coli K-12 MG1655F⁺ (Blattner et al., 1997).

Genetic changes in the dcw gene cluster of the L-form

To identify genetic differences correlating with an altered division process in the cell wall-less L-form, we sequenced completely the dcw region (18.331 bp) from mraZ to lpxC (Fig. 2; division and cell wall synthesis gene cluster). dcw harbors essential genes for cell division and murein synthesis which are conserved in many distantly related eubacterial genomes (Nanninga, 1998; Tamames et al., 2001). In addition, we cross-examined several division-specific genes distributed along the chromosome (zapA, zipA, ftsB (ygbQ), ftsK, and ftsN, and further genes encoding control elements for cell division in E. coli (minCDE, sulA; Margolin, 2003). In total, we analyzed 35 646 bp of the L-form genome. Altered genes were found exclusively within dcw of the L-form affecting the division-specific genes ftsQ, ftsA, ftsW, ftsZ, and the cell wall synthesis genes mraY and murG. All changes are caused by point mutations. None of them affects known promoters (e.g. ftsQp2/Qp1, ftsA1p, ftsZp4, ftsZp3, ftsZp2) retrieved from EcoCyc (Keseler et al., 2005). Figure 2 summarizes the dcw genes examined in this study, and shows the genetic changes in the dcw locus of the L-form.

Altered fts genes of the L-form

The division-specific genes in the L-form dcw are affected differently (Fig. 2). A nonsense mutation results in a premature stop codon in ftsQ. Missense mutations in ftsA and ftsW lead to amino-acid (aa) replacements, and a silent mutation is present in ftsZ.
FtsQ is a bitopic membrane protein (Fig. 3), and previous work from Beckwith and colleagues has shown that its periplasmic extension of 225 aa at the C-terminus is the functionally important domain (Chen et al., 2002). It contains residues critical for spatial localization at the Z-ring. Furthermore, it is required to recruit partner proteins for the division process in normal E. coli. A deletion of 29 C-terminal aa (13% of the extension removed) still allowed the targeting to the constriction site but the cells were unable to divide, as they could not recruit further division partners (Chen et al., 2002). The precise physiological function of FtsQ, like most of the fts genes, is unknown.

Others have speculated that FtsQ is not only required for cell division but could also play a role in cell wall synthesis during the late phase of cell division (Nanninga, 1998; Chen et al., 2002). The precise physiological function of FtsQ, like most of the fts genes, is unknown. Others have speculated that FtsQ is not only required for cell division but could also play a role in cell wall synthesis during the late phase of cell division (Nanninga, 1998; Chen et al., 2002). Recently, FtsQ was shown to harbor a POTRA (polypeptide-transport-associated) domain within its periplasmic extension (aa position 55–126). This domain is discussed to mediate a chaperone function, possibly for the assembly of OM proteins (Sanchez-Pulido et al., 2003).

In the L-form, a nonsense mutation (W132TAG) truncates more than half (64%) of this extension from the ftsQ product (Fig. 3). Whereas the segment important for recruiting and localization is deleted, the POTRA domain remains preserved. As cell division in E. coli requires the correct localization as well as the hierarchical recruitment of division proteins, it is surprising that cell division is not blocked in the L-form. Specifically, the formation of the division subcomplex FtsQLB should be prevented, which has been shown to be conserved among E. coli, Bacillus subtilis and Streptococcus pneumoniae (Buddelmeijer & Beckwith, 2004). As the L-form cells are viable, still can grow, replicate and divide, FtsQ – at least the part lacking from its extension – seems not to be required for division of the wall-less L-form.

Whereas the nonsense mutation suggested a loss-of-function, the missense mutations in the genes ftsA and ftsW (Figs 2 and 4) currently do not allow conclusions with respect to cell division-specific alterations. The missense in the L-form-FtsA was found at a conserved...
position in the derived FtsA proteins of diverse bacteria (Fig. 4), e.g. in *Thermothoga maritima*, *B. subtilis*, and even of a *Mycoplasma*-type species (Zhao et al., 2004). The A116E exchange resides in subdomain 1c of FtsA, very close to a segment specifically required for homodimerization of the actin-like cytoplasmic protein (Carettoni et al., 2003; Rico et al., 2004). The gene *ftsW*, encoding an integral membrane protein with unknown function (Boyle et al., 1997; Mercer & Weiss, 2002) is changed by a D85Y replacement in the N-terminal part at the membrane-periplasm border (Fig. 4). In contrast to FtsA and FtsW, the primary structure of FtsZ is not altered due to a silent mutation in the L-form (Fig. 2). Also the *minCDE* and *sulA* genes are not changed (data not shown) which play a role in the control for Z-ring formation. Thus, the function of FtsZ (Margolin, 2005) seems to be preserved in the L-form.

L-form cell division has been considered to proceed as in *Mycoplasma* which are naturally occurring cell wall-free bacteria (Margolin, 2000). The question was raised whether not suitable for studying the sufficiency of FtsZ, as a model.

### Altered cell wall synthesis genes of the L-form

In addition to the mutations in the *fts* genes, we detected two mutations which affect the cell wall synthesis genes *mraY* and *murG* (Figs 2 and 3). They normally encode enzymes which together catalyze the formation of the lipid-linked disaccharide pentapeptide, lipid II, the precursor required for murein biosynthesis (Nanninga, 1998). The first step of this process involves MraY, an integral membrane enzyme consisting of 10 membrane spanning helices. It catalyzes the binding of UDP-MurNAc-(pentapeptide) to bactoprenol, a membrane-bound C55 isoprenoid lipid, to form the water-soluble building blocks of the murein sacculus for the transport across the hydrophobic barrier of the CM.

### Alignment analysis of FtsA and FtsW from the L-form *Escherichia coli* LW1655F^+^.

The A116E exchange in FtsA is indicated by an arrow. The segment found to play a role in homodimerization is underlined. The D85Y exchange in FtsW is indicated by an arrow. For the alignment analyses the derived primary structures of diverse bacteria were used. The abbreviations of the strains: L, LW1655F^+^; K12, *E. coli* K-12 MG1655; EcO, *E. coli* O157:H7 EDL933; C57, *E. coli* CFT073; Shi, *Shigella flexneri* 2a strain 2547T; Vch, *Vibrio cholerae* KIM; Yep, *Yersinia pestis* CO92; Sty, *Salmonella enterica* ssp. enterica serovar Typhi Ty2; Bap, *Buchnera aphidicola* (Baizongia pistaciae); Bsu, *Bacillus subtilis* subsp. *B. subtilis* strain 168; Psa, *Pseudomonas aeruginosa* (PA01); Nme, *Neisseria meningitidis* strain Z2491; Thm, *Thermotoga maritima* (strain MS88); Sku, *Spiroplasma kinkelii*.

In *Mycoplasma*-like bacteria (C. Hoischen, pers. commun.).
spanning the cytoplasmic loop V (Fig. 3). It suggests that MraY is nonfunctional, or is severely impaired in its function to act together with MurG, an N-acetylglucosaminyl transferase. In the L-form, also MurG is altered by a point mutation. The underlying I119V replacement is probably of minor importance, because position 119 in the MurG primary structure is not conserved among enteric bacteria (data not shown). Notably, MraY and MurG have been selected as priority targets for the development of drugs killing murein-containing bacteria (Zawadzke et al., 2003). Our results indicate that L-form cells can grow without the function of MraY, in contrast to normal *E. coli*.

**Conclusions**

This report represents the first mutation based analysis of a bacterial L-form. It focussed on the genetic background of cell division and cell wall synthesis genes in a bacterial protoplast-type L-form which was derived from *Escherichia coli* (Schuhmann & Taubeneck, 1969). L-form bacteria represent a curiosity in microbiology, and how they divide was open to speculation (Margolin, 2000). Our results suggest that this L-form is able to bypass the function of *ftsQ* and *mraY*, which are absolutely required for cell division and growth in all bacteria with a murein-based cell wall (Nanninga, 1998; Margolin, 2000). Recent experimental studies with *E. coli* indicate that bypass mechanisms and compensatory capacity exist within the bacterial division machinery, that were not anticipated. Studies have shown that subtle changes in the primary structure of cell division proteins can render other division functions dispensable which were believed to be essential [e.g., FtsA can compensate for ZipA (Geissler et al., 2003)]. Moreover, the apparent linearity for recruitment of the components of the division machinery at midcell does not necessarily reflect a temporal order (Goehring & Beckwith, 2005; Goehring et al., 2005). Importantly, the signals used to recruit the division partners seem to be diverse and include protein–protein interactions, regulated assembly of subcomplexes and probably also the recognition of peptidoglycan substrates at the septum (Goehring & Beckwith, 2005; Goehring et al., 2005). In this context and on the basis of our work, we propose that the L-form can be used as a model to further investigate the compensatory capacity and the bypass mechanisms within the bacterial division system. Further experiments are now needed to understand how cell division without concomitant murein sacculus synthesis is accomplished in the L-form. This should be of particular interest to understand whether a pathway exists which enables bacteria to persist without a cell wall, or as cell wall-deficient bacteria, serving as cryptic agents for a variety of human infections. This opinion, and occasional reports on cell wall-free bacteria in medical samples which revert to the walled form upon isolation and cultivation are controversially discussed by microbiologists for more than 50 years (Domingue & Woody, 1997).

As a note of caution concerning the transition of a walled *E. coli* to a protoplast: the mutations determined could reflect the current state of LW1655F+ rather than the primary mutational events leading to the protoplast phenotype. The loss-of-function of *ftsQ* and *mraY* without consequences for viability could represent secondary mutations upon completion of the transition, as the strain has been propagated under laboratory conditions for more than 30 years (Gumpert et al., 2002). Nevertheless, elucidating how this L-form divides will have impact to understand the plasticity of the bacterial cell division and cell wall synthesis machinery in general.

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**References**


Supplementary material

The following supplementary material is available for this article online:

Table S1. dcw-specific oligonucleotides.
Table S2. Gene-specific oligonucleotides.
Table S3. 16S rRNA-specific oligonucleotides.

This material is available as part of the online article from http://www.blackwell-synergy.com