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ABSTRACT

Growing bacterial L-forms are reputed to lack peptidoglycan, although cell division is normally inseparable from septal peptidoglycan synthesis. To explore which cell division functions L-forms use, we established a protocol for quantitatively converting a culture of a wild type *Escherichia coli* K-12 strain overnight to a growing L-form-like state, using the β-lactam cefsulodin, inhibitor of penicillin-binding proteins 1A and 1B. In rich hypertonic medium containing cefsulodin, all cells are spherical and osmosensitive, like classical L-forms. Surprisingly, however, mutant studies showed that colony formation requires D-glutamate, diaminopimelate and MurA activity, all of which are specific to peptidoglycan synthesis. HPLC analysis confirmed that these L-form-like cells contain peptidoglycan, with 7% of the normal amount. Moreover, the β-lactam piperacillin, a specific inhibitor of the cell division protein PBP3, rapidly blocks cell division of these L-form-like cells. Similarly, penicillin-induced L-form-like cells, which grow only within the agar layer of rich hypertonic plates, also require D-glutamate, diaminopimelate and MurA activity. These results strongly suggest that cefsulodin- and penicillin-induced L-form-like cells of *E. coli* – and possibly all L-forms – have residual peptidoglycan synthesis which is essential for their growth, probably required for cell division.

INTRODUCTION

Bacterial L-forms are spherical, osmosensitive variants, isolated from many different species and reported to have no peptidoglycan cell wall, although they grow and divide indefinitely. In contrast, cell division in normal bacteria is indissociable from cell wall synthesis; a septum, consisting of a double layer of peptidoglycan, is laid down at midcell and then split to form the new poles of the two daughter cells. The division of L-forms in the apparent absence of peptidoglycan is thus paradoxical. We wished to explore which cell division functions L-forms require, using *Escherichia coli* K-12 as model.

Septal synthesis is carried out by an assembly of cell division proteins organized in a ring-like, membrane-associated complex surrounding midcell (19, 52). The structural basis of this ring is the FtsZ protein, a bacterial tubulin homologue. In *E. coli* some 15 cell division proteins are known to be recruited into the FtsZ ring.

The study of L-forms goes back to 1935 when Emmy Klieneberger succeeded in establishing a pure culture of the curious mycoplasma-like organism that appeared systematically in cultures of *Streptobacillus moniliformis* (26, 27). It consisted of spherical, osmosensitive cells that grew on plates of hypertonic complex medium containing serum. Klieneberger called the culture "L1" in honor of the Lister Institute in London, where she had emigrated in 1933 when the German authorities fired her as a Jew. Similar morphological variants, also called L-forms, were subsequently isolated from other bacterial species (10, 11).

In 1942 L-forms were found to be resistant to penicillin (40). This provided a convenient tool for isolating L-forms from essentially any cultivable bacterial species. The protocol that emerged was to spread a heavy inoculum of bacteria on a plate of hypertonic complex medium containing serum, broth and penicillin. After a growth period, usually of several weeks, an agar block is cut from the plate, inverted and spread on a plate of the same medium for a new growth period. Such "passages" are repeated serially, often for years, until finally a stable L-form appears, able to grow indefinitely and no longer able to revert to normal morphology when cultivated in the absence of penicillin. Two such L-forms of *E. coli* K-12 are extant, one isolated in 1987 after heavy mutagenesis (38) and the other in 1969 without mutagenesis (43).

There is little information on the genetics of L-forms but it is clear that a number of mutations accumulate during the many passages; the two extant stable L-forms of *E. coli*

K-12, both carry numerous uncharacterized mutations (see Discussion). To study the cell division functions required for the propagation of *E. coli* in the absence of peptidoglycan, we needed a genetically defined lineage. We therefore sought a procedure to convert all cells of a given culture (of known genotype) to the L-form on command, as it were. With appropriate genetic constructs, the resulting L-form cells could then be tested for their ability to divide after depletion of one or another cell divison protein. Using the β-lactam cefsulodin, we developed a protocol that converts an entire population of wild type *E. coli* overnight to viable, growing cells that are osmosensitive and spherical. In the present report we characterize these L-form-like cells. To our surprise, physiological, genetic and biochemical evidence, presented here, showed that they still synthesize 7% of the normal amount of peptidoglycan and that this residual synthesis is essential for their propagation, most likely for cell division.

Wild type *E. coli* can grow in the presence of penicillin only if embedded within the agar layer of a rich, hypertonic plate, where it produces minicolonies of spherical L-form cells (31). However, we present genetic and physiological evidence that this growth, too, absolutely requires residual peptidoglycan synthesis.

We conclude that cefsulodin- and penicillin-induced L-form-like cells of *E. coli* retain the ability to synthesize small amounts of peptidoglycan and that this residual synthesis is essential.

MATERIALS AND METHODS

Bacterial strains and plasmids. With one exception, all experiments presented here were carried out with MG1655 (2) and derivatives obtained by P1 transduction (36) and transformation (42). The exception is the experiment with VIP205, genotype MC1061 [*araD139* Δ(*araA-leu*) Δ(*codB-lacI*) *galK16 galE1 mcrA mcrB1 hsdR2 relA1 spoT1 rpsL150*] (Ω–Km^R-lacI^q-P$_{tac}$)::*ftsZ* (16). The chromosomal alleles introduced into MG1655 (and their description) are: Δ*dapA*::Ery^R (8), *dapB17*::Mu (6); *dapE*::Cm^R (Coli Genetic Stock Center, http://www.cgsc.biology.yale.edu); *gltS murI*::Km^R (12); *murA*::Km^R (5); Δ*mrcA*::Km^R (34), Δ*mrcB*::Km^R (9); Δ*rcsA*::Cm^R, Δ*rcsB*::Cm^R, Δ*rcsC*::Cm^R, Δ*rcsD*::Cm^R and Δ*rcsF*::Cm^R (13); *cpsE3*::Tn*10* (49).

The plasmids used are: pJFK1183H(Km^R) and pUM1Ba(*mrcB*$^+$ Km^R) (35); and pBAD(*murA*$^+$ Cm^R). The latter was constructed by isolating a 1.4 kb *Kpn*I-*Xba*I fragment carrying the *murA*$^+$ gene from pBAD30-Z(Amp^R) (5) and recloning it in pBAD33(Cm^R) (22) digested by the same enzymes.

The altered *gltS* allele permitting efficient D-glutamate uptake was transduced into strain MG1655 *pyrE zib-563*::Tn*10*, selecting Ura$^+$ and screening for cotranductants that were Tc^S and able to grow on glutamate as sole carbon source; one such strain was then transduced to *murI*::Km^R. The *dapB17*::Mu allele was introduced into this *gltS murI*::Km^R strain by cotransduction with *carA*::Tn*10*; the latter allele was then removed by transduction to Arg$^+$ Pyr$^+$. The *murI*::Km^R allele was removed by transduction to *argE*::Tn*10* and screening for D-glutamate prototrophy; one such strain was then transduced to Arg$^+$ Tc^S to produce a *dapB17*::Mu *gltS* strain.

For strains MG1655 *lacY*::Cm^R Δ*mrcB*/pJFK1183H (Km^R) and MG1655 *lacY*::Cm^R Δ*mrcB*/pUM1Ba (*mrcB*$^+$ Km^R), the Km^R cassette in the *mcrB* gene, flanked by two *res* sites, was removed using the non-replicative plasmid pJMSB8 carrying the resolvase (29); this plasmid is propagated in strain S17-1(λ*pir*).

Media and growth conditions. L-form-like cells were grown in M medium, a rich hypertonic medium specially designed for this work. It contains: beef extract (Difco) 3 g/l, Bactopeptone (Difco) 10 g/l, yeast extract (Difco) 5 g/l, NaCl 5 g/l, $MgSO_4$ 0.01 M, sucrose 0.23 M. M plates contained in addition 1.2% Bactoagar (Difco). For routine growth, LB broth (36) was used; for some experiments LB broth was prepared without added NaCl. DAP and

DL-glutamate, when needed, were used at 30 and 100 µg/ml, respectively. Antibiotic concentrations were: chloramphenicol 20 µg/ml, ampicillin 100 µg/ml, tetracycline 10 µg/ml, erythromycin 200 µg/ml, piperacillin 3 or 5 µg/ml, aztreonam 0.1 µg/ml, A22 [S-(3,4-dichlorobenzyl)isothiourea, Calbiochem] 8 µg/ml, cefsulodin (Sigma) 30 µg/ml.

Unless indicated otherwise, all bacterial growth was at 30°C. Liquid cultures were agitated vigorously.

Muropeptide analysis. Total peptidoglycan and the degree and type of cross-linking were measured as described (18, 50). In brief, 250 ml overnight cultures of strain MG1655 grown with aeration in M medium with or without cefsulodin, were centrifuged, resuspended in ice-cold water and dropped into a boiling 6% SDS solution. After boiling for 15 h, the crude sacculi were collected by ultracentrifugation, washed free of SDS with water, and digested with α-amylase for 2 h at 37°C and with pronase for 90 min at 60°C. After the addition of 1% SDS, the sacculi were boiled for 30 min; SDS was then removed by repeated centrifugation-resuspension. The murein samples were digested with the amidase cellosyl. Muropeptides were analyzed after reduction with sodium borohydride by separation on reversed-phase HPLC and quantification of the UV absorption of the muropeptides. The total amount of peptidoglycan was calculated as the sum of the absortion of all muropeptides.

Protein assay. To compare the amount of peptidoglycan in bacteria growing in M medium with and without cefsulodin, we normalized the amount of muropeptides to the amount of protein, evaluated with a DC Protein assay (Biorad). To validate this, we measured the protein concentration relative to the OD_{600}. Bacterial cultures were prepared exactly as for the peptidoglycan assay. After boiling with SDS, the protein concentration was measured. The two cultures had the same protein concentration for a given OD_{600} (220 µg/ml for an OD_{600} of 1).

Microscopy. Bacterial suspensions were placed at 40°C and diluted twofold with a 2% solution of Low Melting Point agarose solution (Gibco) kept at the same temperature; 8 µl of the mixture was placed on a prewarmed slide, 2 µl of a 200 µg/ml solution of FM 4-64 (Molecular Probes) was added and a cover slip was placed on the droplet. Bacteria were examined in a LEICA DMRE2 videomicroscope using a wide field 100x objective fitted with a mercury arc lamp, a red filter (SC4) and a high resolution CoolSnap HQ camera (Photometrics). Each cell was photographed in 35 to 54 focal planes, using a piezo motor PI.

The images were analyzed with Image J 1.36b software (NIH). This program was used to obtain the pictures of Fig. 1 and the cell diameter distribution of Fig. 2.

RESULTS

L-form-like growth in the presence of cefsulodin. In 1958 Lederberg and St. Clair reported that the *E. coli* K-12 strain Y10 made L-form colonies with 10 to 50% efficiency when plated within the agar layer of a hypertonic complex medium containing 1000 U/ml penicillin; no L-form growth occurred on the surface of the plates or in liquid medium of the same composition (31). We reproduced these results with the wild type *E. coli* K-12 strain MG1655 and our rich hypertonic M medium (see Materials and Methods). Growth was observed at an agar concentration of 1.2% but not at 0.6%, and there was no growth on the surface of these plates or in liquid M penicillin medium.

To carry out analyses on L-form cells, it is convenient to propagate them in liquid culture or on the surface of plates to avoid the difficult problem of separating the fragile cells from the agar. We therefore sought an alternative protocol that could produce L-form growth on the surface of M medium plates or in liquid culture. We focused in particular on PBPs 1A and 1B. These enzymes catalyze the polymerization of glycan chains, with lipid-linked disaccharide pentapeptide units as substrate (23). PBPs 1A and 1B are bifunctional enzymes, possessing both transglycosylase (chain lengthening) and transpeptidase (cross-linking) activity. Genetic studies in 1978 established that *E. coli* grows normally in the absence of PBP 1A or PBP 1B but lyses in normal (isotonic) media when both are genetically inactivated (47). The β-lactam cefsulodin specifically inhibits the transpeptidase activity of PBPs 1A and 1B, causing lysis (37). We investigated the possibility of inducing L-form growth on the plate surface using cefsulodin instead of penicillin.

The effect of different concentrations of cefsulodin on the growth of MG1655 on M plates is shown in Table 1. At 30 or 100 µg/ml, mucoid colonies appear overnight and contain only spherical cells. If the osmolarity of the medium is further increased, growth is slowed down considerably. If it is lowered, ultimately the cells no longer form colonies in the presence of cefsulodin; on plates of LB medium to which no NaCl is added, for example, the presence of 30 µg/ml cefsulodin reduces the plating efficiency 200-fold.

We next studied the effect of temperature on the plating efficiency in the presence of cefsulodin. On M plates containing 30 µg/ml cefsulodin the efficiency of plating (e.o.p.), 62% at 30°C (Table 1), dropped to 10% at 37°C and 5% at 42°C. In the following work cefsulodin, when used, was added at 30 µg/ml, and all cultures and plates were incubated at 30°C.

We tested the ability of the spherical, L-form-like cells appearing on M cefsulodin plates to continue growing in the same conditions. A colony was resuspended in M medium and assayed on M plates with or without cefsulodin. The e.o.p. on the former was 25-50% compared to the titre in the absence of cefsulodin. The colonies on the M cefsulodin plate were mucoid and contained only spherical cells whereas those on the M plate without cefsulodin were non-mucoid and the cells in them had reverted to rod-shaped morphology.

A colony from an M cefsulodin plate inoculated into M cefsulodin liquid medium, after a lag period, exhibited exponential growth with a doubling time of 60 min, compared to 30 min in M medium without cefsulodin. Growth during this phase was balanced, as judged by a constant ratio of viable cell concentration to optical density (OD_{600}); at saturation both cultures reached an OD_{600} between 1.0 and 2.0 (data not shown). It is also possible to establish L-form-like growth directly in M cefsulodin liquid medium, using as inoculum cells grown in M medium without antibiotic. Cells growing in M cefsulodin liquid cultures are uniformly spherical (see below).

L-form-like growth in mutants lacking PBP 1A or 1B. PBPs 1A and 1B are specified by the *mrcA* and *mrcB* genes, respectively. The synthetic lethality of *mrcA* and *mrcB* mutations (47) suggests that these proteins are to some degree functionally redundant. To study the effect of genetic inactivation of *mrcA* or *mrcB* on cefsulodin-induced L-form-like cells, we constructed Δ*mrcA* and Δ*mrcB* derivatives of MG1655. As expected, both grew normally in M medium, with rod-shaped morphology. On M cefsulodin plates, the Δ*mrcA* strain, totally lacking PBP 1A, behaved like wild type: overnight it formed mucoid colonies (e.o.p. about 25%) containing only spherical cells. The Δ*mrcB* mutant, in contrast, grew extremely poorly on M cefsulodin plates. It formed visible colonies only after at least four days' incubation, with an e.o.p. of about 0.1; the cells in the colonies were spherical.

β-Lactams inactivate the transpeptidase activity of PBPs but not the transglycosylase (23). The above observations suggest that the transglycosylase activity of PBP 1B is more important than that of PBP 1A for L-form-like growth in the presence of cefsulodin. . A more important role for PBP 1B was also observed when PBP2 or PBP3 was specifically inhibited; under these conditions, cells lysed rapidly in the absence of PBP1B but continued growing for several generations in the absence of PBP1A (14).

Transformation of the Δ*mrcB* strain with a plasmid carrying the *mrcB*$^+$ gene under P_{lac} control restored rapid growth on M cefsulodin plates containing the *lac* operon inducer IPTG (3×10^{-5}M), with an e.o.p. of 0.6 in 24 h. Under these conditions of PBP 1B overexpression, however, the cells in the colonies were rod-shaped or filamentous rather than spherical.

Osmosensitivity and envelope disorganization in L-form-like cells. Classical L-forms are generally osmosensitive. We tested the osmosensitivity of cefsulodin-induced L-form-like cells by subjecting them to an osmotic downshift. A culture of spherical MG1655 cells grown in M cefsulodin medium was plated on twofold diluted LB medium to which no NaCl had been added. It had an e.o.p. of 7×10^{-4}, whereas rod-shaped MG1655 cells, grown in M medium without cefsulodin, had an e.o.p. of 1.0 on this medium.

When an M cefsulodin culture was centrifuged, washed and resuspended in distilled water, there was massive lysis. This was caused by the osmotic downshift, not by the centrifugation, since washing and resuspending in M cefsulodin medium gave 100% recovery. Rod-shaped cells grown without cefsulodin were not affected by washing and resuspension in distilled water.

These results show clearly that cefsulodin-induced L-form-like cells are osmosensitive.

Osmosensitive cells are fragile. To evaluate the degree of spontaneous lysis in our hypertonic M medium, we grew a culture in the presence of the *lac* operon inducer IPTG (5×10^{-4} M), with or without cefsulodin, to see how much β-galactosidase was liberated in the medium. Enzyme levels were low (580 and 750 Miller units, respectively). For osmosensitive spherical cells (culture with cefsulodin), about 20% of the total enzyme activity remained in the supernatant (after 15 min centrifugation at 15000 rpm) or in the filtrate (after passage through a 0.2 μm membrane filter); for rod-shaped cells about 11% remained.

In the course of the above experiments we made a striking observation. The usual β-galactosidase assay includes a permeabilization step (treatment with chloroform and SDS) since the substrate ONPG can diffuse across the outer membrane but not across the cytoplasmic membrane. Although ONPG can be taken up by lactose permease, transport is considerably slower than hydrolysis by β-galactosidase and thus limits the rate of ONPG hydrolysis. In rod-shaped cells growing in M IPTG medium, omission of the permeabilization step gave an apparent specific activity only 12% that of permeabilized cells; this is primarily enzyme released by spontaneous lysis. With spherical cells, in sharp contrast, fully 50 to 60%

of the enzyme activity was detected in unpermeabilized cells, well above the level of lysis. This strongly suggests that the cefsulodin-induced L-form-like cells are permeable to ONPG. This in turn indicates that their cytoplasmic membrane, although intact, is somewhat disorganized.

In Gram negative bacteria the outer membrane, tightly associated with the peptidoglycan layer, is the first barrier protecting the cells against toxic products. In L-forms of Gram negative species, the outer membrane has been variously reported to be absent or present in altered form, called respectively protoplast- and spheroplast-type L-forms (21). Indeed, advantage has been taken of this to establish a protein display, in which proteins anchored in the outer leaflet of the cytoplasmic membrane are exposed at the surface of protoplast-type L-form cells (20).

We tested the sensitivity of our cefsulodin-induced L-form-like cells to three toxic agents which, at low concentrations, are excluded from *E. coli* by the outer membrane: SDS, rifampicin and novobiocin (39). MG1655 grew normally, with an e.o.p. near 1, on M plates containing 3.5% SDS, 5 µg/ml rifampicin or 150 µg/ml novobiocin. Growth was abolished, however, if the plates also contained cefsulodin (e.o.p. $< 2\times10^{-4}$ in all cases). L-form-like cells are thus hypersensitive to these three compounds.

Some L-forms of *E. coli* have been found to be resistant to various bacteriophages (48). We tested our L-form-like cells for sensitivity to phage λ by spotting λ*vir* on a lawn of cells on an M cefsulodin plate. Lysis was observed. On a lawn of MG1655 *lamB*, lacking the λ receptor, there was no visible lysis. Phage λh^{80}*cI*, which uses the TonB-activated FhuA outer membrane protein as receptor, lysed both strains. These observations indicate that the outer membrane proteins LamB and FhuA are present in L-form-like cells and recognizable by the phage.

We conclude that our L-form-like cells have an outer membrane which is in a somewhat disorganized state.

Cell shape and size distribution. We studied the morphology of our L-form-like cells. To see cell contours clearly, the bacteria were stained with FM 4-64, a dye which enters the membrane where it becomes fluorescent. Taking serial pictures in successive focal planes allowed us to get precise measurements of individual cells (see Materials and Methods).

MG1655 cells from an M cefsulodin culture were uniformly spherical (Fig. 1). Cell size was heterogeneous, as has been observed with classical L-forms. The average diameter was 1.33 µm, standard deviation 0.50 µm, with 89% of the cells having a diameter between 0.6 and 1.8 µm (Fig. 2), corresponding to a 27-fold range in volume (0.11 to 3.0 μm^3). Dividing cells revealed that cell division is often asymmetrical, producing daughter cells of unequal size (Fig. 1).

These observations are in sharp contrast to the situation with rod-shaped cells growing in the absence of cefsulodin: cell size was more uniform with less than a threefold range in volume, the average volume was larger (3.2 μm^3), and cell division took place precisely at midcell.

MreB independence and capsule dependence of L-form-like growth. The rod shape of wild type *E. coli* is ensured in part by the actin-like protein MreB (4). This is an essential protein (30), although its precise role has not been clearly established. A specific inhibitor of MreB has been described, the chemical A22 (17, 25).

On M plates A22 inhibits the growth of MG1655. On M cefsulodin plates, in striking contrast, the L-form-like cells are completely resistant to A22 (Table 2).

This strongly suggests that the normally essential actin-like protein MreB, involved in maintaining rod shape, is not required for the growth of spherical L-form-like cells.

Cells growing on M cefsulodin plates form mucoid colonies, indicating overproduction of capsular polysaccharide, which in *E. coli* K-12 consists of colanic acid. The enzymes that carry out this biosynthesis are specified by a group of genes regulated by the RcsBCD stress system (24, 32). We inactivated the synthesis of colanic acid by introducing into MG1655 either a regulatory mutation (Δ*rcsB*, Δ*rcsC*, Δ*rcsD*, Δ*rcsA*, Δ*rcsF*) or a *cpsE*::Tn*10* allele inactivating the structural gene of an enzyme in the colanic acid biosynthetic pathway (49). The resulting strains were all unable to grow overnight on M cefsulodin plates (e.o.p. $< 5\times10^{-4}$), indicating that L-form-like growth on the surface of an M cefsulodin plate requires a protective colanic acid capsule.

These results show that L-form-like growth on the surface of an M cefsulodin plate requires a protective colanic acid capsule.

Growth within the agar layer of an M plate can also protect the cells. We looked to see whether these conditions obviate the requirement for capsule. The MG1655 *cpsE*::Tn*10* mutant was unable to grow within the agar layer of an M cefsulodin plate (e.o.p. < 5×10^{-5}). Thus agar cannot replace the capsule requirement for L-form-like growth.

L-form-like growth requires ongoing peptidoglycan synthesis. Since L-forms are thought to have no cell wall, we attempted to establish L-form growth by blocking a specific step in peptidoglycan synthesis. We first studied an auxotroph for D-glutamate, the second amino acid of the pentapeptide side chain of muramic acid. A D-glutamate auxotroph of MG1655 grew efficiently as rods on M D-glutamate plates (and as spheres on M cefsulodin D-glutamate plates), but it was unable to grow in the absence of D-glutamate (e.o.p. < 10^{-4}, with or without cefsulodin).

We next examined MG1655 derivatives that require diaminopimelate (DAP), the third amino acid of the muramic acid side chain. We constructed *dapB*::Mu and *dapE*::Cm^R derivatives of MG1655 (see Materials and Methods). Both grew efficiently as rods on M DAP plates (and as spheres on M cefsulodin DAP plates), but again neither could grow in the absence of DAP (e.o.p. < 2×10^{-6} for both strains, with or without cefsulodin).

In a final attempt to obtain L-form growth by means of a genetic block to cell wall synthesis, we constructed a strain in which the *murA* gene product can be depleted. This cytoplasmic enzyme catalyzes the first reaction in the synthesis of the muramic acid side chain. We constructed an MG1655 Δ*murA* derivative carrying a plasmid with the *murA* gene under control of the *araBAD* promoter, expressed only in the presence of exogenous L-arabinose. The resulting strain grew efficiently as rods on M plates containing 5×10^{-4} M L-arabinose (and as spheres on M cefsulodin L-arabinose plates), but it was unable to form colonies on M plates lacking L-arabinose (e.o.p. < 2×10^{-5}, with or without cefsulodin).

Although we were unable to induce L-form growth on the surface of M plates when cell wall synthesis was genetically blocked, it was conceivable that the difficulty lay in the initial establishment of L-form-like growth but not in the subsequent propagation of pre-established L-form-like cells. We therefore tested these conditions again, using for inoculum spherical L-form-like cells from cultures pregrown in the presence of cefsulodin.

We prepared liquid cultures of *murI*::Km^R, *dapB*::Mu and *murI*::Km^R *dapB*::Mu double mutant strains in M medium containing cefsulodin, DAP and D-glutamate, and of the

Δ*murA*/*pBADmurA*$^+$ strain in M medium containing cefsulodin and L-arabinose. The cultures, which contained only spherical L-form-like cells, were all assayed on M plates with or without cefsulodin and with or without the supplements (DAP+D-glutamate or L-arabinose). None of the mutants could grow without its supplement, with or without cefsulodin (e.o.p.s all < 10^{-3}).

We conclude that a tight genetic block in peptidoglycan synthesis completely prevents the propagation of L-form-like cells of MG1655 on the surface of M cefsulodin plates. This in turn suggests that these cells have residual peptidoglycan synthesis which is essential for their propagation.

Peptidoglycan in L-form-like cells. To test this hypothesis directly, we assayed the cells for peptidoglycan. We grew MG1655 for about 20 generations in liquid M cefsulodin medium and, in parallel, in liquid M medium without cefsulodin. The cells were harvested and subjected to the procedure for peptidoglycan purification and hydrolysis (see Materials and Methods). HPLC analysis of the muropeptides revealed clearly that the L-form-like cells did indeed contain a low amount of peptidoglycan, about 7% as much as the rod-shaped cells. This estimate may neglect very short glycan chains not cross-linked to higher molecular weight peptidoglycan and thus not pelleted in the ultracentrifugation step (see Materials and Methods).The glycan chains were on the average a third shorter in the L-form-like cells, as estimated by the fraction of anhydrosugars (Table 3). The cross-linking pattern was also somewhat different in the two samples. The muropeptides from the L-form-like cells had more DAP–DAP cross-links and fewer DAP–D-Ala cross-links. The latter, which are D–D cross-links, can be formed by PBPs 1A, 1B, 2, and 3; this presumably accounts for their relative underrepresentation in the presence of cefsulodin, which inhibits the transpeptidase activity of PBPs 1A and 1B. The former, which are L–D cross-links, are not formed by PBPs; L–D cross-linking enzymes have not been identified in *E. coli* (23).

Thus our L-form-like cells must synthetise peptidoglycan, as indicated by the genetic results. The quantitative differences observed in cross-linking and average chain length are probably attibutable to the inactivation of the transpeptidase activity of PBPs 1A and 1B; in fact, the residual peptidoglycan synthesized in the presence of cefsulodin is relatively normal in structure.

Cell division requirements of L-form-like cells. Penicillin G inhibits all PBPs and prevents surface growth of L-form-like cells whereas cefsulodin, which inactivates only PBPs 1A and 1B, does not. It would thus seem that one or more of the PBPs are required for surface growth. Since PBP3 is required for cell division (45) and PBP2 has been implicated in the division of spherical cells (51), we investigated the ability of L-form-like cells to grow in the presence of specific inhibitors of PBP2 or PBP3.

Mecillinam specifically inhibits PBP2 (45 , 46). Spherical MG1655 cells grown overnight in liquid M cefsulodin medium were plated on M cefsulodin plates with or without 3 μg/ml mecillinam. No growth was observed in the presence of mecillinam (e.o.p. $< 1x10^{-3}$), indicating that PBP2 activity is required for L-form-like growth.

Piperacillin and aztreonam are specific inhibitors of PBP3 (=FtsI), which is required for septation (45). Spherical MG1655 cells grown overnight in liquid M cefsulodin medium were plated on M cefsulodin plates with or without 3 μg/ml piperacillin or 0.1 μg/ml aztreonam. Both antibiotics prevented growth of the L-form-like cells (e.o.p. $< 5x10^{-4}$), indicating that growth requires PBP3. Furthermore, aztreonam has been reported not to prevent the recruitment of the final division protein, FtsN, into the septal ring (M. Wissel and D. Weiss, cited in (1)), suggesting that L-form-like growth specifically requires PBP3 transpeptidase activity.

For both spherical and rod-shaped cells the antibiotic concentrations used were the lowest that gave a plating efficiency less than 10^{-3}.

To see whether PBP3 is required for cell division of L-form-like cells, we looked at the effect of piperacillin in liquid M medium. To an exponentially growing culture of MG1655 in liquid M cefsulodin medium we added 5 μg/ml piperacillin. The OD_{600} of the culture continued to increase exponentially at the same rate as in the control for 2.5 generations, then stopped. The concentration of viable cells, in contrast, rapidly stopped increasing, with a total increment of less than 50% (Fig. 3). Microscope examination of the cells 4 h after piperacillin addition revealed large spherical cells and frequent clusters of small spherical granules. After overnight incubation the OD_{600} had not changed and the viable cell count had dropped a mere twofold.

The same experiment was carried out on rod-shaped MG1655, growing exponentially in M medium without cefsulodin. In the presence of piperacillin the OD_{600} increased for 3 generations, although at a slower rate than in the untreated control. The concentration of viable cells increased by about 50%, remained constant for two hours, then rapidly dropped 100-fold. The cells initially formed filaments; during overnight incubation they lysed (data not shown).

These observations confirm that cefsulodin-induced L-form-like cells require PBP2 and PBP3 activity and strongly suggest that the latter is required, as in rod-shaped cells, for cell division.

The FtsZ protein is found in all bacteria, including cell wall-less mycoplasmas. In bacteria with a cell wall, FtsZ forms the midcell ring which, when completed with the other cell division proteins, carries out septum synthesis. To test whether FtsZ is required for division of cefsulodin-induced L-form-like cells, we used strain VIP205, in which the chromosomal *ftsZ* gene has been put under exclusive P_{tac} control; the strain grows only in the presence of an inducer of the *lac* operon (16). On M plates containing 3×10^{-5} M IPTG, strain VIP205 formed normal rod-shaped cells; on M cefsulodin IPTG plates it formed spherical cells (e.o.p. 0.6). In the absence of IPTG, no growth was observed in the presence or absence of cefsulodin (e.o.p. $< 2\times10^{-4}$). Thus the cell division protein FtsZ is required for colony formation on M cefsulodin plates.

Penicillin-induced L-form-like cells. *E. coli* MG1655 is unable to grow on the surface of M plates containing 1000 U/ml penicillin G (e.o.p. $< 2\times10^{-7}$). L-form-like cells of wild type MG1655, pregrown in M cefsulodin medium, are also unable to grow on the surface of M penicillin plates (e.o.p. $<2\times10^{-4}$). The e.o.p. within the agar, however, is high, about 0.5, and the cells in the colonies are spherical; such cells were considered unstable L-forms by Lederberg and St. Clair (1958). We were unable to separate the cells from the agar to test for the presence of peptidoglycan, to evaluate osmosensitivity, etc. We could, however, examine the genetic requirements for this L-form-like growth in the presence of penicillin.

Cultures of *murI*::Km^R, *dapA*:Ery^R and Δ*murA*/pBAD*murA*$^+$ derivatives of MG1655 were grown in liquid M medium containing D-glutamate, DAP or L-arabinose, respectively. They were then plated within the agar layer of M plates containing 1000 U/ml penicillin G, with or without the appropriate growth requirement. The mutants all grew in M penicillin agar when the growth supplement was present (e.o.p. ≥ 0.1), but none could grow in the absence of its requirement (e.o.p. $< 5\times10^{-5}$ in all cases). Penicillin resistant growth within the agar layer also required a colanic acid capsule, as evidenced by the inability of the *cpsE*::Tn*10* mutant to grow in these conditions (e.o.p. < 2×10^{-5}).

The above results indicate that growth within the agar layer of M penicillin plates requires D-glutamate, DAP and MurA activity. This strongly suggests that L-form-like growth in the presence of penicillin (within the agar layer), like that in the presence of cefsulodin (on the plate surface), requires residual peptidoglycan synthesis.

DISCUSSION

Normal bacterial cell division is indissociable from septal peptidoglycan synthesis. In apparent contradiction with this, L-form derivatives of bacteria have been reported to have no peptidoglycan, yet they can grow and divide indefinitely. In the present work we present a protocol for quantitatively converting all cells in a culture of a genetically well defined *E. coli* strain to growing L-form-like cells. This can be done by adding the β-lactam cefsulodin, inhibitor of PBPs 1A and 1B, to a culture of an *E. coli* K-12 strain like MG1655 growing in a rich hypertonic medium such as our M medium. The procedure works both on plates and in liquid culture. Like classical L-forms described in the literature, the cells are spherical, osmosensitive, smaller on the average than rod-shaped cells and more heterogeneous in size. Using appropriate mutants, we found, to our surprise, that the propagation of these L-form-like cells requires D-glutamate, DAP and MurA activity, all specific to peptidoglycan synthesis. Direct measurement revealed that these cells do in fact contain peptidoglycan, about 7% of the amount in rod-shaped cells. Coupled with the genetic results, we conclude that this residual peptidoglycan synthesis is essential.

Like many workers before us, we were unable to find conditions in which wild type *E. coli* is able to establish L-form growth on the surface of hypertonic plates containing penicillin. However, MG1655 can grow within the agar layer of M penicillin plates, producing spherical cells. Again to our surprise, we found that the propagation of penicillin-induced L-form-like cells within the agar layer requires D-glutamate, DAP and MurA activity. This strongly suggests that L-form-like growth within M penicillin agar also requires residual peptidoglycan synthesis. We were unable to assay the peptidoglycan content of these cells. We nevertheless conclude that the propagation of both cefsulodin- and penicillin-induced L-form-like cells requires residual peptidoglycan synthesis.

What is the source of this residual peptidoglycan? In the presence of cefsulodin, rapid L-form-like growth clearly requires PBP 1B transglycosylase activity and PBP2 and PBP3 transpeptidase activity. These enzymes probably account for the residual peptidoglycan synthesis in the presence of cefsulodin. During growth within the agar layer in the presence of 1000 U/ml penicillin G, the cells are likely to express various stress responses. We cannot say at present whether these protect one or more PBPs from total inactivation or permit the expression of alternative (unknown) peptidoglycan synthesizing enzymes.

The L-form-like growth described here has an absolute requirement for colanic acid capsule, the synthesis of which is governed by the RcsBCD system, together with the additional regulators RcsA and RcsF (24, 32). The Rcs stress response is induced when the cell envelope is perturbed (24). It is also induced when PBPs 1A and 1B are specifically inactivated (41), consistent with our observations that cefsulodin treatment causes a general disorganization of both the cytoplasmic and the outer membrane.

What is the evidence that classical L-forms have no peptidoglycan? The initial speculation that L-forms lack a cell wall came from their mycoplasma-like morphology; indeed they were initially thought to be mycoplasmas. Electron microscopy showed in many cases that there is no visible cell wall in L-forms of different bacterial species, including *E. coli* (21). Biochemical analyses of cell wall constituents in L-forms have given variable results, with numerous reports in which muramic acid, DAP, D-glutamate, or glucosamine was or was not detected in extracts of L-forms of various bacteria, usually with little quantification. We are unaware of any published data that eliminate the possibility of 7% residual peptidoglycan in an established L-form. We therefore speculate that a low level of residual peptidoglycan synthesis may be a requirement for the propagation of all L-forms.

Little is known of what mutations L-forms can tolerate. It was recently reported that an established *E. coli* L-form isolated nearly 40 years ago has acquired mutations in several genes required for peptidoglycan synthesis and cell division (44). From the sequence of 36 kb of L-form DNA, the authors deduced that the FtsA, FtsW and MurG proteins have one or two amino acid changes each, the *ftsQ* gene has an amber triplet at codon 132 (of 276 codons), and the *mraY* gene has a frame shift in codon 294 that should produce a protein of 298 amino acids (instead of 360). The functional consequences, in rod-shaped cells, of the missense mutations and of the truncation of MraY are unknown. The truncated FtsQ protein would almost certainly be non-functional for cell division in rod-shaped cells, although a low level of amber suppression could provide the 22 molecules of intact FtsQ estimated to be required for division (7). Further characterization of this classical L-form should establish clearly whether or not the cells carry out residual peptidoglycan synthesis and, if they do, whether it is essential for their propagation.

What should be called an L-form has been discussed since 1939, when it was shown that many bacterial species gave rise to forms similar to the L1 culture that Klieneberger had isolated from *S. moniliformis*. Since 1942 the methodology for establishing L-forms routinely involves numerous passages on complex hypertonic penicillin plates over an extended period of time. The first growing cells obtained, unstable L-forms, are spherical and osmosensitive, and they revert to normal morphology in the absence of penicillin. After further passages, often extending over several years, stable (non-reverting) derivatives are obtained, and some authors have suggested that only these should be called L-forms (28). Others, however, have presented convincing evidence that stabilization is a secondary event which simply prevents the reconstitution of a normal cell wall in the absence of penicillin but does not affect L-form growth (31).

What then is an L-form? In the absence of a recognized authority empowered to establish such definitions, the wisest course is to describe clearly the origin and cultivation of the organisms used, whatever name they go by. This, unfortunately, is not always the case in the L-form literature. In the present work, to avoid confusion, we have called our spherical, osmosensitive cells "L-form-like".

The L-form-like growth of *E. coli* described here, whether induced by cefsulodin or penicillin, requires residual peptidoglycan synthesis amounting, in the former case, to 7% of that of wild type cells. This raises the question of the function of this peptidoglycan. The situation is in some ways reminiscent of the paradox of chlamydial species, which are reputed to have no cell wall yet seem to require peptidoglycan synthesis, probably for cell division (33). The amount of peptidoglycan in L-form-like *E. coli* cells is far too little to form a sacculus covering the entire cell (53). Although techniques are not presently available for locating this peptidoglycan within the cell, the following arguments suggest that it may be at the division site of the spherical cells. PBP3, which is specific to the septation process, is required for the propagation of cefsulodin-induced L-form-like cells, and when it is inhibited by piperacillin, there is a rapid block of the viable cell count (Fig. 3). The transglycosylase activity of PBP 1B is required for rapid growth of the L-form-like cells, and this protein has been shown to interact directly with PBP3 (3); PBP 1B has also been implicated in cell division under certain conditions (15). The central cell division protein FtsZ is required for the propagation of L-form-like cells. PBP2, although not normally a cell division protein, has been implicated in the division of spherical cells (51), and it is required for L-form-like growth. The simplest hypothesis to account for these observations is that cell division in cefsulodin-induced L-form-like cells, and possibly in all L-forms, takes place, as in rods, by means of peptidoglycan synthesized in the division plane and indispensable for cytokinesis.

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Table 1. Growth, cell morphology and colony aspect on cefsulodin plates

	cefsulodin concentration (µg/ml)				
	0	**10**	**30**	**100**	**200**
e.o.p.[a]	≡1.0	0.92	0.62	0.10	0.001
cells	rods	rods	spheres	spheres	spheres
colonies	non-mucoid	non-mucoid	mucoid	mucoid	mucoid

[a]Efficiency of plating compared to the titre on an M plate without cefsulodin. Overnight cultures in liquid M medium were diluted and spread on the surface of M plates containing cefsulodin at the indicated concentrations. Plates were incubated 24 h at 30°C.

Table 2. MreB-independence of L-form-like growth		
e.o.p.[a]	**– A22**	**+ A22**
– cefsulodin	º1.0	$<10^{-4}$
+ cefsulodin	0.6	0.5

[a]A culture of MG1655 in M medium was assayed on four types of M plates, with or without cefsulodin (30 µg/ml) and with or without A22 (8 µg/ml). Plates were incubated for 24 h.

Table 3. Analysis of muropeptides in L-form-like cells

	Rods[a]	Spheres[a]
Muropeptides/protein[b]	370x10^3	25x10^3
% DAP-DAP crosslinkage	5.7	13
% Total dimers	32	29
DAP-DAP dimers/total dimers	0.18	0.38
% anhydro muropeptides	9.5	> 14.5
Average chain length	10.6	6.9
% Total cross-linking	37	35

[a]MG1655 cells were grown for 20 generations in liquid M medium without cefsulodin (rods) or with 30 µg/ml cefsulodin (spheres). Cells were harvested and peptidoglycan was extracted and analysed (see Materials and Methods).

[b]Total muropeptide was calculated by integrating all peaks after HPLC separation; total protein was measured as described in Materials and Methods.

Figure legends

Figure 1. L-form-like cells of *E. coli* MG1655. Cells were stained with FM 4-64, a fluorescent membrane dye (see Materials and Methods). Each image was taken in the focal plane giving the maximum diameter.

Figure 2. Diameter distribution of L-form-like *E. coli* MG1655. In all, 248 cells were measured.

Figure 3. Growth of L-form-like cells in the presence of piperacillin. An exponentially growing culture of strain MG1655 in M cefsulodin medium was separated into two parts. At time 0, piperacillin (5 µg/ml) was added to one of them. At the indicated times, the OD_{600} of each culture was measured (left) and the cultures were diluted in M medium and assayed on M cefsulodin plates (right). Plates were counted after 24 h incubation. Squares: culture without piperacillin; circles: culture with piperacillin.

Figure 1 (Joseleau-Petit *et al.*)

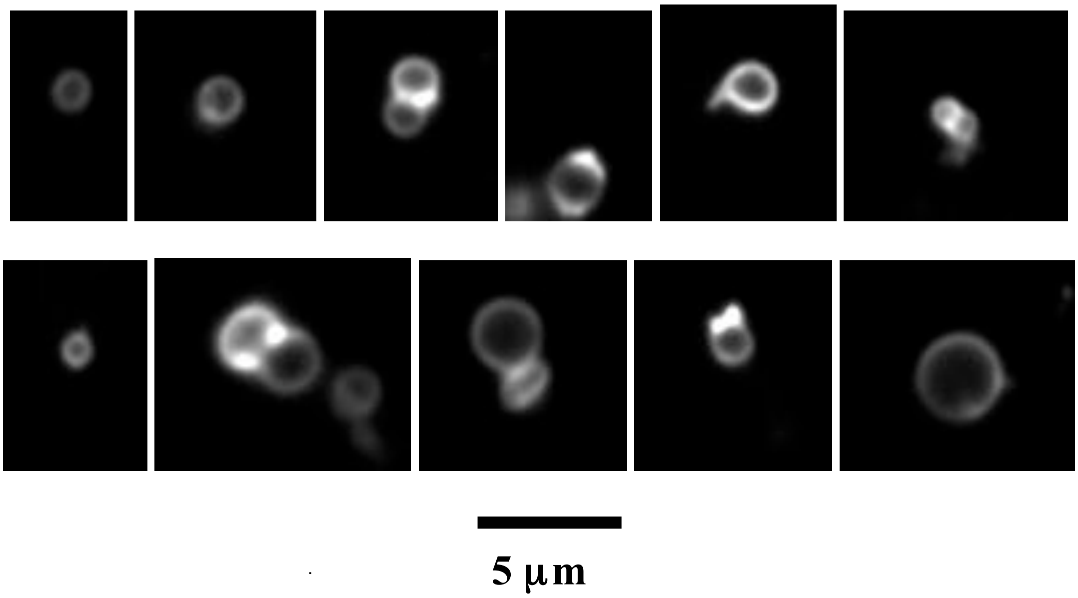

Fig. 2 (Joseleau-Petit *et al.*)

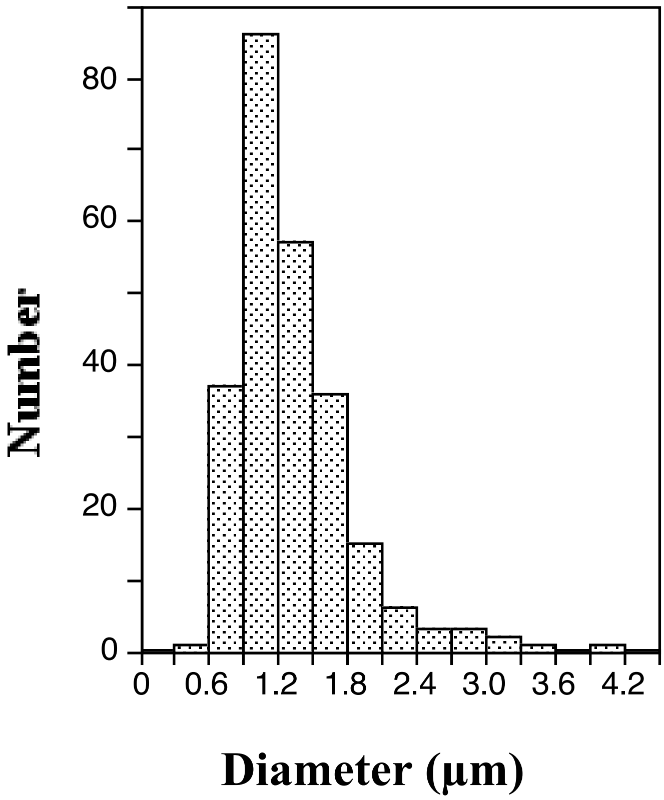

Figure 3 (Joseleau-Petit *et al.*)