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GUEST COMMENTARY

Reforming L Forms: They Need Part of a Wall After All?[▽]

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One of the joys of science is seeing old problems solved. Just as satisfying is the pleasure of seeing long-unanswered questions revitalized by the application of new technologies or simply by someone reinvestigating the problem with fresh eyes. In this issue, Joseleau-Petit et al. (5) provide us the opportunity to share this second kind of enjoyment by resurrecting the rather moribund subject of *Escherichia coli* L forms along with the question of how bacteria survive and flourish after losing their sturdy peptidoglycan cell wall. These authors describe a new technique for converting, at will and with ease, any strain of *E. coli* into an L-form-like variant, and they characterize some of the physiological requirements for creating and maintaining this unusual form. The major surprise is that these cells cannot survive without a remnant of peptidoglycan, which may be required for proper cell division.

L-form bacteria have a long history, beginning (as the authors note in a nice introductory retrospective) with the work of Emmy Klieneberger in 1935, who named the cells in honor of the Lister Institute of London. Several reviews summarize this early work (3, 7, 12), and a more recent article updates the historical literature on L forms, the semantic concerns about how to refer to these variants, the generation and study of several such organisms, and the pathogenic implications of this kind of life (4). Briefly, L forms are bacteria that once possessed cell walls but which have acquired the ability to grow without this rigid exoskeleton. The loss may be permanent (stable L forms) or temporary, so that some may regain their wall and grow normally (unstable L forms). Until now, the overwhelming impression has been that such cells make no peptidoglycan or, at the very least, that they need not do so. The findings of Joseleau-Petit et al. demand that this view be reevaluated for gram-negative L forms.

One problem with the accepted view is that the transition from bacterium to L form should be commonplace, requiring only that the cell be in an isotonic environment and that peptidoglycan synthesis be inactivated. However, great effort is often required to generate and maintain *E. coli* L forms (6), including incubating the cells in complex media in the presence of high concentrations of penicillin, growing them as embedded colonies in a specific percentage of agar, and passaging them multiple times for several years. Why is it so hard to generate L forms and why can they not be created in liquid media?

Joseleau-Petit et al. (5) can now explain why this has been so difficult. First of all, they introduce a procedure to generate "L-form-like" cells from any *E. coli* lineage. The conversion technique is straightforward, consisting of a single overnight incubation in a rich hypertonic medium in the presence of the β-lactam cefsulodin. This antibiotic inhibits the transpeptidase activity of the major peptidoglycan-synthesizing enzymes, penicillin-binding proteins (PBPs) 1a and 1b (2, 8). In hypertonic media, the cells become spherical, osmosensitive, and heterogeneous in size, traits associated with L forms. Unlike their historical forbears, however, these survivors form colonies on an agar surface and propagate in liquid media.

Why does this undemanding protocol succeed? The answer is that the procedure finally achieves a proper balance between the set of reactions to be inhibited versus those that must be retained. Figure 1 summarizes the basic findings. PBPs 1a and 1b are bifunctional enzymes, having a transglycosylase domain that polymerizes glycan chains and a transpeptidase domain that incorporates these polymers into the existing bacterial sacculus by cross-linking their peptide side chains. If the transpeptidases of PBPs 1a and 1b are inactivated by cefsulodin (Fig. 1B), then L-form-like cells arise. The same is true for cells lacking PBP 1a (Fig. 1C), but mutants missing PBP 1b die when exposed to cefsulodin (Fig. 1D). Thus, to create L-form-like cells the cross-linking abilities of these PBPs must be inactivated and the transglycosylase activity of PBP 1b must remain intact. This strongly implies that a peptidoglycan polymer is required for L-form survival (see below).

The reason previous procedures were not as successful is because they included high concentrations of a β-lactam, usually penicillin, that inhibited the transpeptidase activities of numerous PBPs. But some of these other PBPs are essential for L-form survival. Like PBPs 1a and 1b, PBPs 2 and 3 have two domains, a transpeptidase and one domain of ill-defined function. Inhibiting the transpeptidase of either PBP 2 (Fig. 1E) or PBP 3 (Fig. 1F) is lethal for L-form-like cells. This suggests not only that L-form survival may require a glycan polymer but that this polymer may have to be cross-linked by these secondary PBPs. Earlier protocols ran afoul of this requirement because they inhibited all transpeptidation.

To prove conclusively that peptidoglycan synthesis was required for the survival of L forms, Joseleau-Petit et al. removed the supply of peptidoglycan precursors. Inhibiting any of three different cytosolic steps in peptidoglycan synthesis destroyed L-form growth, supporting the contention that the complete loss of peptidoglycan is not tolerated. A corroborat-

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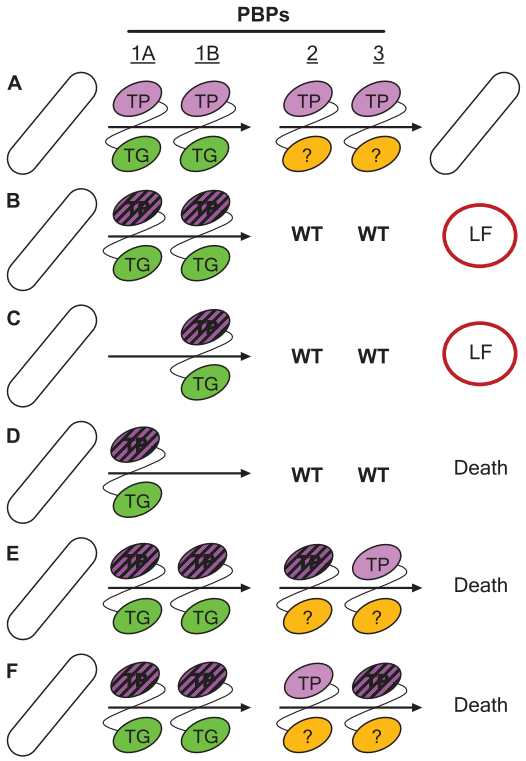

FIG. 1. PBP requirements for creating *E. coli* L forms, according to Joseleau-Petit et al. (5). Rod-shaped cells (left) are treated with β-lactam antibiotics to inactivate different PBPs (represented by two connected ovals), resulting in the propagation of the original rod-shaped cell or an L-form-like cell (LF) or in the death of the organism. PBPs 1a and 1b are composed of a transpeptidase domain (TP, purple oval), which can be inactivated by the addition of cefsulodin (thick black stripes), and a transglycosylase domain (TG, green oval). PBPs 2 and 3 are composed of a transpeptidase domain linked to a domain of uncertain function (orange oval). WT indicates that these proteins are present and uninhibited. (A) Untreated wild-type cells give rise to normal rod-shaped *E. coli*. (B to F) Cefsulodin-treated cells. (B) Wild-type *E. coli*. (C) Mutant deleted for the PBP 1a gene. (D) Mutant deleted for the PBP 1b gene. (E) Wild-type cells treated with amdinocillin to inhibit PBP 2. (F) Wild-type cells treated with piperacillin or aztreonam to inhibit PBP 3.

ing piece of evidence is that the L forms retain 7% of the normal amount of peptidoglycan, the composition of which is surprisingly normal. This finding is exceptional because L forms were assumed to have no peptidoglycan because they were spheroidal, suggesting the absence of a restraining cell wall. However, Sharp did report that a well-established L form of *Proteus vulgaris* retained ~16% of its cell wall muramic acid (10). These older results are intriguingly close to those reported here (5) and imply that the continued presence of peptidoglycan may be a common trait among gram-negative L forms. The existence of this residual peptidoglycan should be interpreted cautiously, though, because it was isolated by repeated ultracentrifugation, and it is possible that additional, shorter glycan chains might also still be present.

The authors suggest that the residual peptidoglycan may help promote cell division. Consistent with this supposition is that L forms do not grow if PBP 3, a component of the divisome, is inhibited (Fig. 1F). However, neither do they survive in the absence of PBP 2 (Fig. 1E), which is more commonly associated with cell elongation, so the relevance of this piece of evidence is uncertain. Depleting the essential division protein FtsZ also inhibits L-form growth, though one might expect this, since division of some sort must obviously take place. Support for the importance of cell division also comes from the partial sequence of a stable *E. coli* L form that carries multiple mutations in cell division genes (11), though it is not known if the mutations are primary (required for L-form creation) or secondary (arising during 40 years of subculture).

Questions. As with any truly satisfying discovery, the work of Joseleau-Petit et al. raises more questions than it answers while at the same time making it possible to attack them. One intriguing subject is how these L forms grow on an agar surface or in liquid media, while classical L forms grow most easily embedded in agar. One explanation is that surface growth depends on a transpeptidase that is inactivated by penicillin selection, though even "within-agar" growth depends on the synthesis of peptidoglycan and colanic acid. This might also inform the long-standing issue of how the cell wall evolved in the first place. It seems unreasonable to require that a rigid exoskeleton spring into place fully formed. More likely, a semistable intermediate preceded the highly cross-linked wall, and an obvious candidate would be a secreted carbohydrate that stabilized the cells in some environments. Cross-linking this polymer would be the next logical step for creating the ordered wall we now observe.

Of course, there are many more questions. Do established *E. coli* L forms retain a requirement for peptidoglycan synthesis? Where does the residual peptidoglycan come from? Might this system be exploited to study the polymerization activities of PBPs 1a and 1b? How does *E. coli* recover its rod shape after growing as a spherical entity, and might this phenomenon be used to probe how cells attain specific morphologies? Why is it that L forms can live without MreB but cells making normal peptidoglycan cannot? Why is it that *E. coli* L forms need a transglycosylase, but another group of bacteria without peptidoglycan, the chlamydia, require transpeptidases (1, 9)? Some of these are new questions, and others have regained life with this work.

Last thoughts. Joseleau-Petit et al. are careful to distinguish the cells they have created from classical L forms by calling their new cells "L-form-like." This seems unnecessarily cumbersome, and I, for one, am willing to call them L forms, thereby establishing a specific, operational definition of that term. These may need to be classified further, for example, as class I (can revert) and class II (cannot revert). Thus, cells created by the procedure of Joseleau-Petit et al. would constitute the minimum class I condition and would form a baseline from which we could characterize additional L-form states. Regardless of what we eventually call them, this work should place the investigation of L forms on a much firmer foundation and should revive interest in, and reinvigorate the study of, this interesting mode of prokaryotic existence.

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