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Cell Growth of Wall-Free L-Form Bacteria Is Limited by Oxidative Damage

Highlights
- The cellular levels of ROS are increased when cell wall synthesis is blocked
- Oxidative damage is a serious impediment to growth of wall-deficient L-forms
- Reduction of ROS levels promotes L-form growth
- L-forms provide new insights into the mode of action of cell wall antibiotics

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In Brief
Although many bacteria are capable of switching into a cell-wall-deficient L-form state, the molecular basis of the transition is still poorly understood. Kawai et al. show that the cellular levels of ROS are abnormally increased when cell wall synthesis is blocked and that counteraction of ROS production leads to improved L-form growth.

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Cell Growth of Wall-Free L-Form Bacteria Is Limited by Oxidative Damage

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SUMMARY

The peptidoglycan (PG) cell wall is a defining feature of the bacterial lineage and an important target for antibiotics, such as β-lactams and glycopeptides. Nevertheless, many bacteria are capable of switching into a cell-wall-deficient state, called the “L-form” [1–3]. These variants have been classically identified as antibiotic-resistant forms in association with a wide range of infectious diseases [4]. L-forms become completely independent of the normally essential FtsZ cell division machinery [3, 5]. Instead, L-form proliferation is driven by a simple biophysical process based on an increased ratio of surface area to cell volume synthesis [6, 7]. We recently showed that only two genetic changes are needed for the L-form transition in Bacillus subtilis [7]. Class 1 mutations work to generate excess membrane synthesis [7]. Until now, the function of the class 2 mutations was unclear. We now show that these mutations work by counteracting an increase in the cellular levels of reactive oxygen species (ROS) originating from the electron transport pathway, which occurs in wall-deficient cells. Consistent with this, addition of a ROS scavenger or anaerobic culture conditions also worked to promote L-form growth without the class 2 mutations in both Gram-positive B. subtilis and Gram-negative Escherichia coli. Our results suggest that physiological compensation for the metabolic imbalance that occurs when cell wall synthesis is blocked is crucial for L-form proliferation in a wide range of bacteria and also provide new insights into the mode of action of antibiotics that target the bacterial cell wall.

RESULTS

The ispA Mutation Suppresses Cell Lysis during Protoplast Growth

We previously showed that protoplasts of B. subtilis, derived by stripping the cell wall with lysozyme in the presence of an osmo-protective agent such as sucrose (Figure 1A, a), fail to proliferate in this state [7]. However, mutants able to proliferate, called “L-forms,” can be selected, and this requires a combination of two kinds of mutations [5, 7]. Class 1 mutations are of several different types, but all work by generating excess amounts of cell membrane, which drives spontaneous shape changes and ultimately proliferation (Figure 1A, b). The original class 2 mutation, which lay in a gene called ispA [5], seemed to work by stabilizing the proliferating L-forms and preventing them from lysing [7] (Figure 1A, c and d). For reasons that remain unclear, mutations that block cell wall precursor synthesis have a class 1 phenotype (i.e., generate excess cell membrane), and this is convenient because these mutations simultaneously prevent the cell wall from being restored.

To improve our understanding of the effects of the ispA mutation on cell lysis, we took advantage of recently developed microfluidic methods [8]. In channels of the microfluidic system, protoplasts are constrained into a near-typical rod-shaped morphology, with approximately similar width to that of wild-type walled cells. Figure 1B (and Movie S1) shows a mixture of B. subtilis protoplasts containing a repressible Ppxyl-murE construct (acts as a class 1 mutation) with ispA−; red cells expressing mCherry) or without (ispA+; unlabelled cells) an ispA mutation trapped in the channels. The ispA− mutant protoplasts (red cells) mainly grew well over many hours in L-form medium in the absence of xylose. In contrast, ispA+ protoplasts (unlabelled cells) frequently lysed after only a limited amount of growth (94% of ispA+ [n = 36] and 35% of ispA− protoplasts [n = 23] resulted in cell lysis in similar experiments). Thus, protoplasts in which peptidoglycan (PG) synthesis is inhibited tend to lyse even in the absence of L-form-like shape changes and cell division, and this cell lysis is suppressed by an ispA mutation.

Reduction of Electron Transport Chain Activity Promotes L-Form Growth

IspA catalyzes the formation of farnesyl pyrophosphate (FPP) in the polyprenoid synthetic pathway [3] (Figure 2A). This pathway leads to the formation of two lipid molecules: heptaprenyl diphasphate (HPP), required for synthesis of menaquinone (MQ), which is involved in the electron transport chain (ETC) system, and undecaprenyl pyrophosphate (UPP), required for synthesis of the precursors for peptidoglycan (lipid II) and wall teichoic acid. If the ispA mutation works through one of these
In previous experiments selecting de novo L-form variants, we repeatedly found that the most robust colonies tended to have mutations in the *ispA* gene [5, 7]. To find out whether mutations in genes other than *ispA* would support L-form growth, we performed a screen in cells containing a second copy of *ispA*. A transposon mutant library was made in cells containing *P*<sub>xyr-murE* (class 1 mutation) grown in the walled state (presence of xylose). L-forms were selected from the mutant library on plates containing sucrose as an osmoprotectant (but no xylose) and the cell division inhibitor, 8j. Six independent transposon mutants were selected, checked by backcrossing, and the sites of transposon insertion were determined by sequencing. Two mutations lay in each of the *ndh* and *qoxB* genes, and single insertions were found in *ctaB* and *mhqR* (Figures 2D and 2E). As shown in Figure 2A, three of these genes encode products involved in the ETC system: *ndh* encodes a major NADH dehydrogenase [12]; *qoxB* encodes cytochrome aa<sub>3</sub> quinol oxidase subunit I [13]; *ctaB* encodes heme O synthase [14]; and *mhqR* encodes a transcriptional repressor for genes induced by the thiol-specific oxidative and/or electrophile stress response [15]. Figures 2D and 2E show the enabling effects of these mutations on L-form growth. Taken collectively, these results suggest that L-form growth, under the conditions we normally use, requires a reduction of ETC activity, and the identification of an *mhqR* mutation further suggests that reactive oxygen species (ROS) originating from the ETC pathway prevent L-form growth. For reasons that are not yet clear, L-forms generated by either *ispA* or *mhqR* mutations were able to grow in liquid medium, whereas the others would only grow on solid agar plates (data not shown).

Abnormally Increased Cellular ROS Levels in Protoplasts Are Reduced by Switching into the L-Form State

In general, all aerobic organisms use oxygen as the terminal electron acceptor for efficient energy production. However, ROS is also generated as a by-product through the metabolism of molecular oxygen, and this causes damage to nucleotides, proteins, and lipids [16, 17]. The cell therefore has various genetic systems to respond to oxidative stress. To investigate whether the oxidative stress response was induced in protoplasts, we compared the gene expression patterns of protoplasts (*P*<sub>xyr-murE* with and without xylose) to those of walled cells (*P*<sub>xyr-murE* with xylose) and L-forms (*P*<sub>xyr-murE* *ispA*<sup><small>+</small></sup>, without xylose) using microarrays. The results showed that the transcription of 103 genes was specifically induced in protoplasts (Tables S1 and S2). Many of those genes (43 genes) have roles against various stresses, including resistance to oxidative and electrophile stress (13 genes), cell envelope stress (12 genes), and heat shock (6 genes). As shown in Figure 3A (see also Figure S1A), strong induction of genes belonging to the PerR regulon, which is induced by the
peroxide-induced oxidative stress [18], was detected in protoplasts. In comparison, many essential genes or functions, such as DNA replication and protein synthesis, for the growth of normal walled state were downregulated in protoplasts (Table S1). The stringent response (Figure S1B), which is induced by amino acid starvation or other stresses [19], may be largely responsible for these downregulation effects. To confirm induction of the oxidative stress response in protoplasts, we examined expression of the katA promoter using a PkatA::gfp fusion [20]. The katA gene encodes a vegetative catalase and is part of the PerR regulon. Figure 3B shows a mixture of exponentially growing wild-type walled cells and an overnight culture of protoplasts (unlabelled) and L-forms (Pspo::ispA, no xylose) in the absence of exogenous oxidant (Figure 3E), suggesting lipid peroxidation by endogenous oxygen radicals. Importantly, the fluorescent shift was largely suppressed in L-forms carrying an isiA mutation (Pspo::isuA, no xylose) (Figures 3F and 3G). Thus, the cellular ROS levels are indeed increased in protoplasts, but they are suppressed by reduction of the ETC activity via inhibition of the PG precursor pathway. See text for details.

To assay more directly for ROS production in protoplasts, we took advantage of a fluorescent fatty acid analog, C11+-BODIPY581/591, which has been used as an indicator of oxidative damage to lipids, i.e., lipid peroxidation [21, 22]. The probe is incorporated into membranes, and the fluorescent properties in the red range of the visible spectrum (emission maximum 595 nm) in fluorescence microscopy shift to the green range (520 nm) upon free radical–induced oxidation. In a control experiment with wild-type B. subtilis strains, BS115 (Wt; Pspo::murE), LR2 (ispA; Pspo::isuA), and BS116 (Pspo::isuA, no xylose) (Figure 3D, i) after treatment with H2O2, green fluorescence was readily detectable in protoplasts, cells, green fluorescence was evident within most cells, and the staining was patchy and irregular (Figure 3D, ii). In contrast to the walled cells, green fluorescence was readily detectable in protoplasts (Pspo::murE, no xylose) in the absence of exogenous oxidant (Figure 3E), suggesting lipid peroxidation by endogenous oxygen radicals. Importantly, the fluorescent shift was largely suppressed in L-forms carrying an isiA mutation (Pspo::isuA, no xylose) (Figures 3F and 3G). Thus, the cellular ROS levels are indeed increased in protoplasts, but they are suppressed by reduction of the ETC activity via isiA mutation. Consistent with the idea that the increased cellular ROS levels in protoplasts originate from the ETC pathway, strong induction of various genes involved in the tricarboxylic acid (TCA) cycle was detected in protoplasts by microarray experiments (Figures S1C and S1D).

**Oxidative Stress Response Genes Are Required to Support L-Form Growth**

As described above, microarray experiments showed specific induction of genes in the PerR regulon and other genes for resistance against oxidative stress in protoplasts (Figure S1A).
However, in many cases, the expression levels in L-forms were also significantly higher than those of walled cells. We wondered whether the stress response genes might be needed to protect cells against oxidative stress during L-form growth. To test this, we inserted an IPTG-dependent promoter in front of four genes or operons encoding antioxidant systems, katA (main vegetative catalase) [23], sodA (superoxide dismutase) [24], bshB1/bshA (bacillithiol synthesis) [25], and zwf (glucose 6-phosphate dehydrogenase; for NADPH generation) [26], in \( P_{\text{MurE ON}} \) or L-forms (LR2; \( P_{\text{MurE ON}} \) ispA\(^*\), no xylose) states. See also Figure S1A and Table S1. However, in many cases, the expression levels in L-forms were also significantly higher than those of walled cells.

**Figure 3. Increased ROS Production in Protoplasts and Its Suppression by an ispA Mutation**

(A) Expression patterns of several PerR regulated genes cultured in the walled (green; strain BS115; \( P_{\text{PerR}} \)-murE, 2% xylose), protoplast (yellow and red; strain BS115; \( P_{\text{PerR}} \)-murE, 2% or no xylose), or L-form (blue; strain LR2; \( P_{\text{PerR}} \)-murE ispA\(^*\), no xylose) states. Note that the thioredoxin system is essential for viability in walled cells and that \( B. subtilis \) lacks a glutathione (GSH) system. In the walled state, none of the genes or operons encoding antioxidant systems, katA, sodA, bshB1, and bshA were significantly expressed (Figure S1B, F). Thus, several antioxidant systems seem to be crucial for L-form proliferation in \( B. subtilis \).

**Reduction of ROS Promotes L-Form Growth in E. coli**

We have previously reported that L-forms of the Gram-negative bacterium \( E. coli \) do not require an ispA-like mutation for proliferation on L-form plates (containing sucrose as osmoprotectant and fosfomycin, an inhibitor of the PG precursor pathway) [3]. Nevertheless, since the growth of \( E. coli \) L-forms is apparently much slower than that of \( B. subtilis \), we wondered whether this was again due to oxidative damage. If so, then treatment of cells with a ROS scavenger such as reduced GSH might improve the growth of \( E. coli \) L-forms. \( E. coli \) walled cells were streaked on L-form selective plates with and without GSH (Figure S1B, F). In the absence of GSH, discrete L-form colonies were barely visible after 3 days of incubation, though a lawn of visible colonies.
emerged after about 5 days (Figures S2A and S2B). In contrast, in the presence of GSH, significant L-form growth was seen within 3 days (Figure 4B, i; Figure 4C, i), supporting the idea that a reduction of cellular ROS levels promotes E. coli L-form growth. If the cellular ROS levels are increased through the metabolism of molecular oxygen in the ETC pathway in E. coli cell-wall-deficient cells, then anaerobic culture should also promote the growth of L-forms. Strikingly, under anaerobic conditions, significant E. coli L-form growth was seen within 3 days even in the absence of GSH (Figure 4B, ii; Figure 4C, ii). We also examined the effects of an ispA mutation on B. subtilis L-form growth under anaerobic condition and found that ispA mutation was no longer required for L-form growth when oxygen was depleted (Figures S2C and S2D).

**DISCUSSION**

We have proposed that L-form proliferation may provide insights into an ancient mechanism used in primordial cells before the invention of the cell wall [3, 7, 28]. In this report, we have found that the cellular ROS levels are abnormally increased in cell-wall-deficient cells and that a reduction of cellular ROS levels by repression of the ETC activity, addition of a ROS scavenger or anaerobic culture, promotes the growth of wall-free L-forms in both Gram-positive and Gram-negative bacteria. Therefore, oxidative damage could be an important impediment to L-form growth in a wide range of bacteria.

Why should the L-form transition or growth in the absence of cell wall result in increased oxidative damage? Cell wall synthesis is probably a major drain on cellular resources under normal conditions, so a block in cell wall synthesis probably leads to major changes in cell metabolism. Uridine 5’-diphospho-N-acetyl-D-glucosamine (UDP-GlcNAc), which is an essential cell wall precursor for both lipid II and WTA synthesis (Figure 2A), is generated from fructose-6-phosphate via central carbon metabolism, through the action of the glmS, glmM, and gcaD gene products. The glmS riboswitch is a ribozyme that self-cleaves upon binding glucosamine-6-phosphate, the product of the enzyme encoded by glmS [29]. Inhibition of PG synthesis would result in increased cellular levels of glucosamine-6-phosphate, and the subsequent repression of glmS could increase glycolytic flux due to a reduction in utilization of fructose-6-phosphate for UDP-GlcNAc synthesis. This could, in turn, result in an increase of cellular pyruvate levels and stimulate flux into the TCA cycle. Consistent with this, our results suggest that the stimulation of ROS production in wall-deficient cells is most likely due to an increase in TCA cycle flux (see Figures S1C and S1D), leading to increased synthesis of NADH and FADH₂, which are the major substrates for the ETC pathway. The subsequent stimulation of ETC flux results in an increase of ROS generation as a by-product of the metabolism of molecular oxygen. Importantly, Kohanski et al. [30] have proposed a model that bactericidal antibiotics, including cell wall antibiotics, work at least in part by stimulating ROS production through a burst of NADH consumption by the ETC pathway, although this is currently controversial [31–33]. Nevertheless, our results suggest that physiological compensation for the metabolic imbalance that occurs when the normal large flux to cell wall synthesis is blocked is crucial for the proliferation of cell-wall-free L-form bacteria. Apart from the importance for understanding early forms of cellular life, the ability to grow without a cell wall in L-forms also provides new insights into the mode of action of antibiotics that target the bacterial cell wall.

**EXPERIMENTAL PROCEDURES**

Experimental Procedures are described in the Supplemental Information.

**ACCESSION NUMBERS**

The accession number for the microarray data reported in this paper is ArrayExpress: E-MTAB-3380.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, two figures, two tables, and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2015.04.031.
AUTHOR CONTRIBUTIONS
Y.K., R.M., and J.E. designed the experiments. Y.K., R.M., L.J.W., P.D.-C., and T.O. performed the experiments. Y.K., R.M., and T.O. analyzed the data. Y.K. and J.E. wrote the manuscript.

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