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REVIEWS

Review

# Cell Wall Deficiency as a Coping Strategy for Stress

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**The cell wall is a surface layer located outside the cell membrane of almost all bacteria; it protects cells from environmental stresses and gives them their typical shape. The cell wall is highly conserved in bacteria and is the target for some of our best antibiotics. Surprisingly, some bacteria are able to shed their wall under the influence of stress, yielding cells that are cell-wall-deficient. Notably, wall-deficient cells are flexible and are able to maneuver through narrow spaces, insensitive to wall-targeting antibiotics, and capable of taking up and exchanging DNA. Moreover, given that wall-associated epitopes are often recognized by host defense systems, wall deficiency provides a plausible explanation for how some bacteria may hide in their host. In this review we focus on this paradoxical stress response, which provides cells with unique opportunities that are unavailable to walled cells.**

## Highlights

Diverse stresses can lead to the formation of cell wall-deficient cells.

Bacteria can reside in eukaryotic systems by becoming cell wall-deficient.

β-Lactam antibiotics can promote a cell wall-deficient life style.

Wall deficiency may facilitate horizontal gene transfer.

## Against All Odds: Life without the Cell Wall

Bacterial cells are enveloped by a complex cell wall which has been instrumental for their evolution into the most abundant non-viral life form on earth. The envelope maintains cell shape, provides structural rigidity and protection from osmotic pressure, and forms the barrier between the bacterium and its environment [1–3]. Given its protective role, the cell wall and its biosynthetic enzymes are targets for some of the best known antibiotics [4–6]. Although the cell wall is a vital structure for bacteria, several species can be induced to produce cells that propagate without their wall [7–11]. Typically, this was done in a rather artificial way by growing strains on osmoprotective media in the presence of high levels of antibiotics targeting the enzymes required for cell-wall synthesis [12–15]. More recently it has become clear that many different groups of bacteria can relatively easily adopt a wall-deficient state, for instance by stimulating certain metabolic pathways or by exposing cells to osmotic stress conditions [16]. Furthermore, it was demonstrated that wall deficiency may be triggered inside eukaryotic host cells [17]. This has led to an increased interest in wall-deficient cells, as this could explain how bacteria shelter inside a host or become insensitive to drugs that target the cell wall synthesis pathway [18,19]. In this review, we focus on these peculiar cells and the consequences of their wall-deficient life style.

## Cell Wall Formation in Bacteria

A major factor that allows bacteria to withstand a wide range of challenging conditions lies in their possession of a stress-bearing cell wall [20]. The cell wall is a dynamic structure that must change continually during cell growth, and its constituents can be altered in parallel with changing environmental conditions [21–23]. Peptidoglycan (PG) is the major component of the cell wall, and its synthesis involves the activity of protein complexes at the cell membrane that cooperatively build and incorporate PG precursors into existing glycan strands [2,6,24–27]. Synthesis of the PG monomer, lipid II, occurs in the cytoplasm and at the surface of the inner leaflet of the lipid bilayer. Flipping of lipid II to the exterior of the cell makes the substrate available to glycosyltransferases that extend the glycan strands, and transpeptidases that create the peptide crosslinks [2,27–29]. The overall structure, sometimes called a sacculus, comprises a single molecule that envelopes the cell and maintains cell shape [30]. Monoderms (traditionally called Gram-positive bacteria) have a thick PG layer exterior to their cell membrane. In contrast, diderm (Gram-negative) bacteria typically have a thin PG layer but are enveloped by a second lipid bilayer called the outer membrane [31]. Recent work has shown that the outer membrane provides a significant degree of structural support to cells, perhaps explaining why they can operate with a relatively thin PG layer [3]. Other polymers that are found in the cell wall matrix include teichoic acids (anionic glycopolymers) in monoderm bacteria and lipopolysaccharides in diderms [32,33]. Both types of bacteria may additionally be enveloped by a proteinaceous 'S-layer'

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[34,35] or a 'capsule', usually of polysaccharide [36,37]. As can be concluded from its high sugar and amino acid content, synthesis of the cell wall is a major investment for cells. For those interested in more detailed information on cell wall synthesis and its regulation, we refer to some excellent reviews on this topic [27,38–40].

## Transient Cell Wall Deficiency as a Stress Response

Bacterial cells are constantly challenged with a range of stresses in their natural environment. For instance, free-living bacteria have to cope with fluctuating environmental conditions, phages and other competing organisms, while cells in a host are confronted with the host's immune system, sometimes combined with high levels of antibiotics administered for treating infections [41–44]. β-Lactam antibiotics are broadly used in the clinic and include penicillin derivatives, carbapenems, cephalosporins, and monobactams. These compounds target the catalytic enzymes involved in cell wall synthesis on the outside of the cell, which are collectively referred to as penicillin-binding proteins (PBPs) [4,45,46]. β-Lactam antibiotics act by irreversibly binding to the active site of transpeptidases, which often leads to bacteriolysis [47]. Recent work revealed that the actual lysis induced by these antibiotics in *Streptococcus pneumoniae* is caused by the relocation of lytic enzymes from the extracellular space devoid of PG to the layer containing PG. The degradation of PG makes the cell wall thinner, ultimately leading to bulging of the cells and catastrophic cell lysis [46]. Although explosive cell lysis is often considered the default response of bacteria when exposed to β-lactam antibiotics, other bacteria, such as *Bacillus subtilis* and *Staphylococcus aureus*, show no bulging, and the induction of prophages can be responsible for any occurring lysis [17,48]. Given that such prophages are common in nature, this mechanism of killing by such antibiotics may be widespread.

It has become increasingly clear that cells have a wide range of defense mechanisms to cope with various stresses, including mechanisms to tune gene expression, the ability to produce compounds targeting phages or other organisms, flagellar systems to escape from stressful conditions, or systems to build multicellular structures such as biofilms, amongst others [23,49–51]. Notably, some stressors may cause cells to adopt a (transient) cell wall-deficient life style. For example, catastrophic bulging and cell death triggered by β-lactam antibiotics is often prevented in osmotically balanced conditions, and also does not happen in most Gram-negative bacteria. Instead, these bacteria tend to lose their PG layer and as a consequence adopt a spherical morphology, known as a spheroplast [19,52]. These spheroplasts remain intact due to the stress-bearing outer membrane [3,53]. This was observed in a range of pathogenic bacteria, including *Pseudomonas aeruginosa*, *Vibrio cholerae*, *Enterobacter cloacae*, and *Klebsiella* species [19,52]. This shows that antibiotic-induced spheroplast formation is widely conserved in Gram-negative bacteria. Notably, *E. coli* was not as tolerant as the other species, and it typically lysed when exposed to β-lactam antibiotics. Importantly, removal of the wall-targeting agents allowed these cells to switch back to their walled state. This indicates that the formation of spheroplasts is an adaptation that allows cells to tolerate exposure to antibiotics targeting the cell wall. Given that these spheroplasts are metabolically active, this response is different from the formation of persisters, which also tolerate high antibiotic levels but are dormant cells with an intact cell envelope [54].

While the responses of cells to cell-wall-targeting antibiotics are the best known examples that lead to wall deficiency, it was recently found that hyperosmotic stress caused some filamentous actinomycetes, for example, *Kitasatospora viridifaciens*, to shed their wall and form so-called S-cells (or Stress-induced cells) [16] (Figure 1). Hyperosmotic stress conditions are probably common for soil bacteria in nature. Possibly as an adaptation to this problem, *K. viridifaciens* and about 10% of other filamentous actinomycetes tested were found to have the ability to extrude S-cells from their hyphal tips in their environment. The S-cells were unable to proliferate without their cell wall. Instead, they increased in size over time and started to accumulate patches of PG at the cell surface. S-cells showed a disordered, nonuniform pattern of cell wall regeneration whereby wall material was often found to detach from the cell surface. However, S-cells were still found to be fairly robust, with approximately one third of the cells surviving a sudden osmotic shock. Ultimately, S-cells were able to rebuild their cell wall and revert to the canonical filamentous mode of growth, indicating that the cell wall-deficient

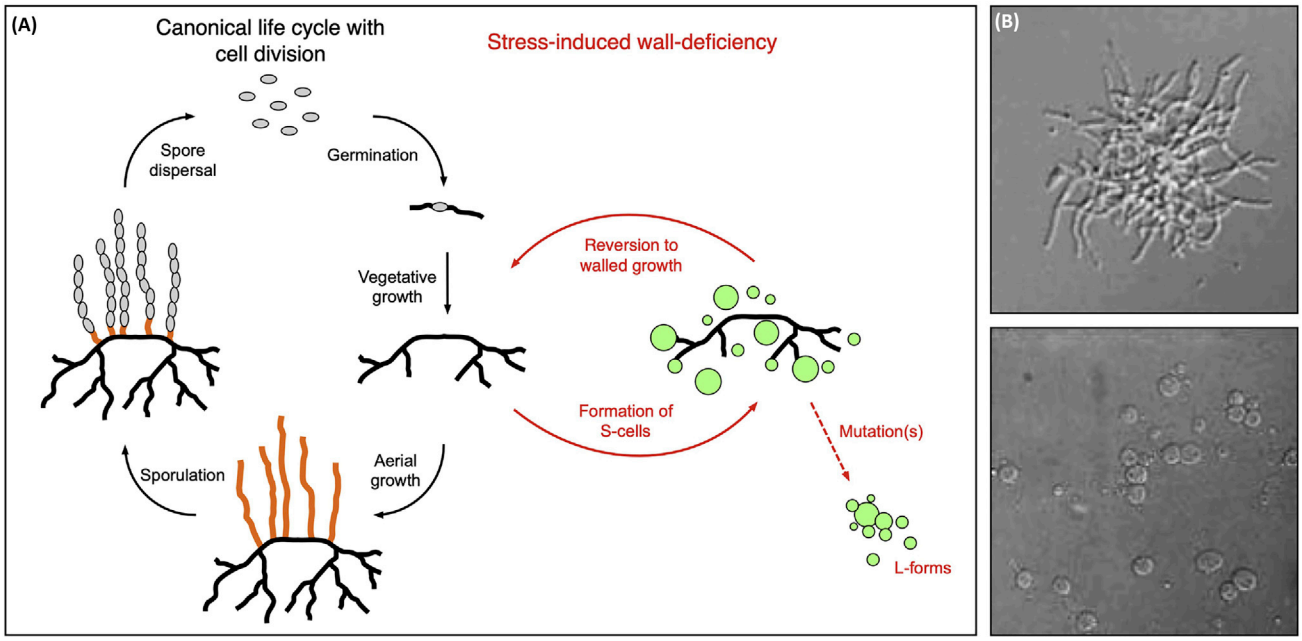


**Figure 1. Stress-Induced Wall Deficiency in Filamentous Actinomycetes.**
(A) The canonical life cycle of the filamentous actinomycete *Kitasatospora viridifaciens* involves the formation of mycelial networks after spores have germinated in a favorable environment. Following a period of vegetative growth, aerial hyphae are formed that later develop into spore chains. When *K. viridifaciens* is exposed to hyperosmotic conditions, S-cells (indicated in green) are extruded. These S-cells are able to revert to the canonical mycelial mode of growth. Prolonged exposure to the stress conditions may lead to the accumulation of mutations and the formation of L-forms, which are able to proliferate without the cell wall. (B) Morphology of mycelium (top) and L-forms (bottom) of *K. viridifaciens*.

(CWD) state of S-cells is transient [16]. Altogether, these results show that exposure of bacteria to various stresses can lead to the formation of wall-deficient cells. How these stresses mechanistically lead to wall deficiency is unknown.

## From a Transient to a Permanent CWD Life Style

While many bacterial cells can transiently shed their wall cell, some bacteria have adopted a permanent wall-deficient life style. Tenericutes, including, for instance, *Mycoplasma* and *Ureaplasma* species, are bacteria that thrive without their wall [55]. The Tenericutes persist inside an osmotically stable environment within the eukaryotic host, which presumably allowed their ancient ancestors to dispose of their cell wall. The ability to be CWD is not restricted to bacteria that colonize eukaryotic hosts. Many diverse groups of bacteria can be forced to grow without a cell wall as so-called L-form cells. L-forms, called after the Lister institute, were first observed by Emmy Klieneberger who worked on the rod-shaped Gram-negative bacterium *Streptobacillus moniliformis* [7]. Klieneberger observed a wide range of spherically shaped cells in her cultures, which she first thought were formed by a symbiont. However, further investigation revealed that these spherical cells had the remarkable property of propagating without their cell wall and were in fact the same species as *S. moniliformis* [56]. It remains unknown how *S. moniliformis* is able to switch between growth with and without a cell wall.

L-form growth can be induced by interrupting the process of cell wall synthesis, for instance by exposing walled bacteria to high levels of lytic enzymes or wall-targeting antibiotics [57,58]. Such inducing conditions often have to be used for several weeks before a so-called 'stable' L-form strain is obtained, which keeps proliferating in the wall-deficient state even in the absence of the inducing agents [15].

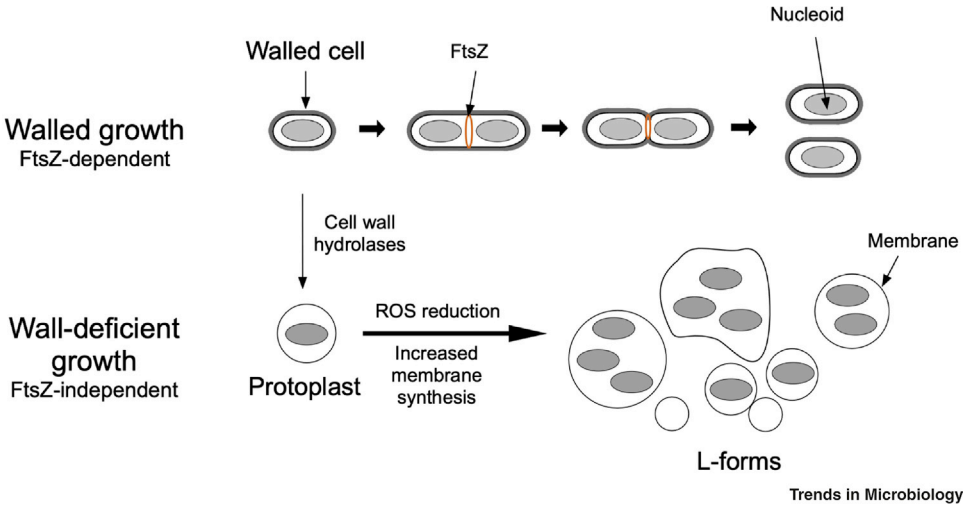


**Figure 2. Contrasting Modes of Growth in the Presence and Absence of the Cell Wall.**
Walled unicellular bacteria divide by binary fission, which is usually dependent on the conserved FtsZ protein that assembles into a ring-like structure at mid-cell. FtsZ acts as a scaffold for the cell division machinery that ultimately leads to the formation of two identical daughter cells that each inherit a chromosome. Cell wall hydrolases, such as lysozyme, can degrade the cell wall, leading to the formation of protoplasts. These protoplasts are unable to proliferate due to oxidative damage caused by the accumulation of reactive oxygen species (ROS). Mutations that reduce ROS levels, combined with an upregulation of membrane synthesis, enables growth without the cell wall as L-forms. Proliferation of L-forms is independent of FtsZ and yields asymmetric progeny containing various numbers of chromosomes. Please note that, in this schematic, a monoderm bacterium is shown.

In the past decade we have begun to understand the biology of L-forms by systematic studies on these cells using the genetically tractable bacterium *B. subtilis*. Using this model system, it was established that three things need to happen to convert a walled bacterium into an L-form that efficiently proliferates in the wall-deficient state (Figure 2). First, cells have to escape from the sacculus, which can be achieved by the activity of endogenous enzymes (autolysins) or exogenous lytic enzymes [17]. This escape is also promoted by mutations damaging the cell wall structure at division sites [59]. As a consequence, protoplasts are extruded more efficiently, due to the weakened spots in the cell wall. Following escape from the sacculus, cells have to cope with increased oxidative stress levels [11]. We think that this oxidative stress results from the excess sugar – normally used for building the wall – flowing through glycolysis and the tricarboxylic acid (TCA) cycle. This increased flux leads to the formation of NADH and FADH2, which are major substrates of the electron transport chain (ETC) that uses oxygen as the terminal acceptor. As a by-product of the ETC pathway, reactive oxygen species (ROS) are formed, causing oxidative stress and ultimately killing the wall-deficient cells [60]. Consistent with these findings, growth under anaerobic conditions or on gluconeogenic substrates, blocking glycolytic sugar uptake, damping down glycolysis or the ETC, or upregulating oxidative stress genes, all enabled growth of *B. subtilis* L-forms [8,11,60]. These findings have been extended to various other Gram-positive bacteria and were further corroborated by the observation that *Enterococcus faecium*, which lacks key components of the ETC, did not require any of these enabling mutations or growth conditions [60].

The last step that is required for efficient proliferation without the cell wall is an upregulation of membrane synthesis. This can be achieved either directly by upregulating components of the fatty acid biosynthesis pathway or as an indirect consequence of a reduction in cell wall synthesis itself [58,61]. This is usually the case when cells are exposed to antibiotics that target cell wall synthesis (for instance D-cycloserine or phosphomycin). Potentially, the increased flux through glycolysis mentioned above generates excess acetyl-CoA, which is the key substrate for fatty acid synthesis, as well as the TCA cycle, but this remains to be confirmed. Nevertheless, the increased membrane

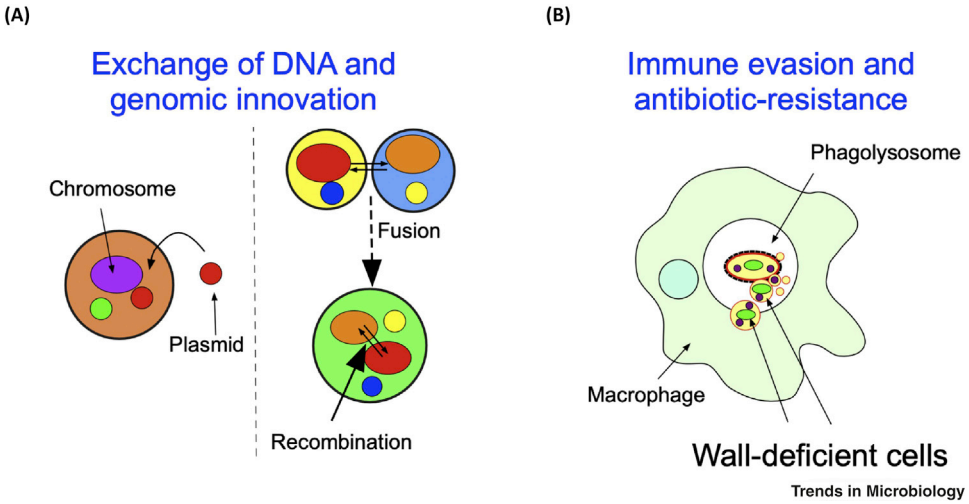


**Figure 3. Consequences of a Wall-Deficient Life Style.**
(A) Wall-deficient cells can take up DNA from the environment or via fusion with other wall-deficient cells. This can lead to exchange of DNA via recombination and hence to genomic innovation. (B) In the host, walled bacteria can be converted to wall-deficient cells by the activity of host lytic enzymes such as those produced in macrophages. These wall-deficient cells are insensitive to wall-targeting antibiotics and are able to evade some elements of immune recognition. Thus, wall deficiency provides opportunities to bacteria that are not readily available to walled cells.

synthesis leads to an excess of surface area relative to volume, which is thought to spontaneously drive shape changes leading to progeny cell formation [61]. In support of this model, large membrane vesicles without any cellular content can generate smaller vesicles when certain fatty acids are added to them [62]. The idea that the division of L-forms, like large membrane vesicles, is simply driven by biophysical effects, is supported by experiments showing that this does not require the cell division machinery that is essential for division in the walled state. Indeed, FtsZ has been deleted or inhibited in various organisms without any noticeable effects on L-form proliferation [8,10,58].

The pioneering studies on the molecular cell biology of L-form growth of *B. subtilis* have been experimentally tested in various other bacteria, including *E. coli*, *S. aureus*, *Listeria monocytogenes*, and *Corynebacterium glutamicum* [58]. Notably, the underlying principles allowing cells to proliferate without their wall appeared to be similar. These findings are supportive of the suggestion that the mode of proliferation of L-forms is ancient and perhaps reminiscent of how primordial cells proliferated long before the cell wall evolved [63]. The capacity of some modern cells to proliferate via L-forms suggests that it provides a selective advantage under at least some conditions. Indeed, there have been a number of observations that different stresses can cause bacteria to adopt an L-form state. In addition to the examples of antibiotic-induced L-forms observed in clinical samples, other examples include prolonged exposure to osmotic stress in filamentous actinomycetes [16], cryogenic or nutrient availability stress in *Mycobacterium bovis* [64,65], and heat stress in *E. coli* [66]. These examples illustrate that the conversion of walled bacteria to the L-form state may serve as a coping strategy to endure environmental insults.

## Consequences of a CWD Life Style

While it seems intuitive to designate CWD cells as fragile or delicate, wall deficiency may also provide cells with unique opportunities that are unavailable to walled cells (Figure 3). As mentioned before, the absence of the cell wall makes these cells resistant to wall-targeting agents. In fact, cell-wall-targeting antibiotics even promote the conversion to a wall-deficient life style [17]. Penicillins and cephalosporins, like several other β-lactam antibiotics, are produced by microbes residing in the

soil [67]. The formation of wall-deficient S-cells by filamentous actinomycetes, which also inhabit soil environments, may allow these bacteria to avoid killing and have an advantage over competitors that are unable to form such cells. This strategy may be particularly relevant for filamentous bacteria given their inability to rapidly move to escape from unexpected danger.

In clinical settings, wall deficiency may allow bacteria to survive antibiotic treatments. Recent evidence has unambiguously demonstrated that Gram-positive bacteria can survive a β-lactam treatment within mammalian macrophages [17]. They do so by converting into L-forms, a process that is stimulated by the activity of host lytic enzymes, such as lysozyme. This was not only observed with macrophages, but also in an animal model [17]. The malleable state of CWD cells may even contribute to dispersal of cells inside the host via narrow pores or tight junctions that are inaccessible to walled cells. It is also likely that wall-deficient forms are less readily detected by the innate immune system since they lack various molecules recognized by pathogen-associated pattern-recognition receptors, especially teichoic acids and PG [68,69]. Wall deficiency may thus also allow cells to escape innate immune killing in a state that is simultaneously insensitive to wall-targeting antibiotics. Crucially, after antibiotic treatment is terminated, these cells (particularly Gram-negative bacteria) can efficiently revert to their walled state and reinitiate an infection of the host, which could explain why certain bacteria cause recurring infections [52,70].

Wall deficiency may also allow cells to acquire new genetic information. For many decades, scientists have used lytic enzymes to degrade the PG-based cell walls of bacteria in order to introduce foreign DNA [71,72]. Likewise, L-forms and S-cells can also take up DNA and may thus contribute to horizontal gene transfer [73,74]. Furthermore, we hypothesize that CWD cells can rapidly evolve due to cell–cell fusion, which has been well documented for protoplasts [75,76]. Finally, CWD cells could also genetically change without the actual uptake of DNA. This is perhaps most evident in reverting S-cells of actinomycetes, which revealed colonies with a wide range of different phenotypes. These phenotypic differences are in part explained by dramatic chromosomal rearrangements and lesions, perhaps due to transposase activity triggered by osmotic stress [16,77]. And while these massive genetic changes will often be deleterious, they may in some cases lead to the evolution of new beneficial traits [78]. Taken together, these examples demonstrate that wall deficiency provides cells with various capabilities that are unavailable to walled cells.

## Concluding Remarks

In the past decade we have witnessed a dramatic increase in our understanding of the biology of CWD bacteria. However, there are still many unresolved questions, and perhaps the most intriguing one is what their functional relevance is in the environment or in association with other organisms. This is an inherently difficult question to answer, and at this stage we can only speculate about their role. Filamentous actinomycetes are present in soil environments but are also abundantly found as endophytes in plants [79]. Interestingly, little attention has been paid to the morphology of actinomycetes in plants. Plant sap contains high levels of osmolytes, which are presumably sufficient to sustain cells in a wall-deficient state. Indeed, there are several reports that L-forms can reside inside plants [80,81]. Accordingly, the natural ability of actinomycetes to form wall-deficient cells may allow these organisms to extrude such cells upon entering the plant – potentially in roots – and travel through the plant to other sites. Their malleable shape may further facilitate their passing through narrow spaces.

Wall-deficient cells of pathogenic bacteria have also been found numerous times in clinical samples [82–85]. Yet, the interpretation of these findings is complicated, given that it is often difficult to unambiguously distinguish wall-deficient cells from eukaryotic tissue, or even to assign such cells to a particular bacterial species. Most of these studies were done in the premolecular era and well before their biology had been investigated. It was recently shown that bacteria are converted into wall-deficient cells in macrophages, whereby they retain their viability [17]. While cells in a CWD state are likely not virulent, it enables bacteria to remain opaque to the immune system or survive antibiotic treatment. Furthermore, the potential ability to revert to the walled state, in principle, enables these

bacteria to reinfect their host. A better understanding of this morphological plasticity would open up routes to effective strategies to combat pathogens that may exploit wall deficiency as a mechanism to reside in the host. For instance, wall-deficient cells are most likely more sensitive to membrane-active agents [18]. The challenge would be to effectively target such drugs to macrophages or other immune cells in which these wall-deficient cells shelter. With the availability of an advanced cell biology toolbox for most bacteria, and high-end sequencing approaches, we now have the means to definitively test their role in recurrent or chronic infections (see Outstanding Questions).

One final question that we would like to bring forward is what this dramatic stress response – loss of the cell wall – tells us about the evolution of the structure. Given that the cell wall contains sugar-rich polymers, one may speculate that the cell envelope initially evolved for storage of excess nutrients. Acquiring the ability to synthesize and tether simple polysaccharides to the cell surface would provide a competitive advantage, allowing cells to explore new areas containing fewer resources or survive periods of starvation stress. Simultaneously, simple surface-associated polysaccharides could have started to provide protection to environmental fluctuations, such as desiccation. Further studies of wall-deficient variants of modern cells should continue to provide insights into possible evolutionary pathways of primordial life.

**Outstanding Questions**

How do stresses mechanistically lead to CWD life styles?

What is the relevance of CWD cells in pathogenesis?

What does transient loss of the wall tell us about the evolution of the wall? How did the wall evolve in the first place?

Does transient cell wall deficiency provide an opportunity for developing new antimicrobial strategies?

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