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REVIEW
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Cell wall deficiency – an alternate bacterial lifestyle?

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Abstract

Historically, many species of bacteria have been reported to produce viable, cell wall deficient (CWD) variants. A variety of terms have been used to refer to CWD bacteria and a plethora of methods described in which to induce, cultivate and propagate them. In this review, we will examine the long history of scientific research on CWD bacteria examining the methods by which CWD bacteria are generated; the requirements for survival in a CWD state; the replicative processes within a CWD state; and the reversion of CWD bacteria into a walled state, or lack thereof. In doing so, we will present evidence that not all CWD variants are alike and that, at least in some cases, CWD variants arise through an adaptive lifestyle switch that enables them to live and thrive without a cell wall, often to avoid antimicrobial activity. Finally, the implications of CWD bacteria in recurring infections, tolerance to antibiotic therapy and antimicrobial resistance will be examined to illustrate the importance of greater understanding of the CWD bacteria in human health and disease.

INTRODUCTION

The cytoplasmic membrane of most bacterial species is enclosed by a rigid structure called the 'cell wall', which is composed of a complex lattice-like arrangement of peptidoglycan molecules that provides shape determination and maintenance, structural strength, and protection from the environment [1–4]. In Gram-negative bacteria, the outer membrane (OM) also contributes to the mechanical load-bearing properties of the cell wall [5]. Interestingly, many bacteria can exist as cell wall deficient (CWD) variants that have lost some or all of their cell wall peptidoglycan and yet remain viable in the CWD state [6, 7]. Due to the loss of the rigid cell wall, CWD bacteria appear spherical, ovoid or pleiomorphic and can be sensitive to osmotic pressure and mechanical lysis [8–10].

It is probable that CWD variants were first reported in the mid-1800s [11]; however, it wasn't until the early 1900s that they were considered to be more than just a laboratory artefact, when in 1935 Emmy Klieneberger-Nobel described round or pear-shaped bodies in a culture of *Streptobacillus moniliformis* that she incorrectly attributed to be symbiotic bacteria [12]. She termed these 'L-forms' in honour of the Lister Institute where she worked at the time of their discovery [12]. Subsequent research conducted by Louis Dienes, among others, determined that the L-forms that Klieneberger-Nobel believed to be symbionts were actually the same species that were spontaneously switching between different morphological states, so that the *Streptobacillus moniliformis* culture was a mixture of L-forms and bacillary cells [13]. Dienes later posited that these spontaneous morphological variations were not a characteristic of only this species, but that under the right conditions all bacteria had the capacity to transition into and revert back from a CWD state [7]. Indeed, CWD variants have been described for many bacterial species, including the important human pathogens *Acinetobacter baumannii* [14, 15], *Escherichia coli* [16–24], *Klebsiella pneumoniae* [18, 25, 26], *Listeria monocytogenes* [27], *Mycobacterium tuberculosis* [28], *Pseudomonas aeruginosa* [29, 30], *Salmonella* sp. [31], *Staphylococcus aureus* [24, 27, 32] and *Vibrio cholerae* [33, 34].

Historically, a range of nomenclature has been used to refer to CWD bacteria, including L-forms, L-phase, L-organisms, gymnoplasts, round forms, round bodies, cysts, ovoid cells, S-cells and spherical cell morphotypes. Some commentators have restricted the use of the term L-form to refer only to CWD cells that can proliferate whilst in the CWD state [35]. However, the term is applied loosely, often used to describe CWD variants where no evidence of proliferation has been provided. In this review, we will use the collective term CWD to refer to all CWD variants of normally cell-walled bacteria. It is important to note that CWD

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Abbreviations: CWD, cell wall deficient; OM, outer membrane; PBP, penicillin binding protein; ROS, reactive oxygen species.

variants do not include species in the bacterial class *Mollicutes*, such as *Mycoplasma* spp. and *Phytoplasma* spp., that have evolved to no longer require a cell wall [36, 37].

There appears to be a remarkable degree of variation in the properties of CWD bacteria. They can differ in the degree of cell wall material present and as such are classified as either 'spheroplasts' that retain some peptidoglycan, or 'protoplasts' that are devoid of all peptidoglycan. CWD bacteria can also be broadly classified as either stable or unstable (transient). Stable CWD bacteria have acquired genetic mutations that lock them in the CWD form, whereas unstable CWD bacteria are considered to be genetically identical to their parental cell and have retained the ability to transition back to the normal cell-walled form upon removal of the inducing agent [13, 19, 20, 24, 35, 38, 39].

Our own interest in CWD bacteria began with the observation that when *Pseudomonas aeruginosa* was treated with high concentrations of antibiotics that inhibit peptidoglycan biosynthesis, most cells rapidly transitioned to a CWD spherical cell morphotype. These CWD cells rapidly reverted to the normal bacillary form when the antibiotic was removed or degraded [29]. Similar rapid CWD transitions have also been reported in a wide range of other clinically relevant Gram-negative bacteria when treated with cell wall targeting antibiotics [19, 40–42].

Another differentiator of CWD bacteria is their relative fragilities. It is widely considered that CWD cells, in particular those referred to as L-forms, are fragile and highly sensitive to osmotic pressure and require specialized osmoprotective culture conditions to prevent lysis [9, 43–45]. However, in our own experience with *Pseudomonas aeruginosa*, CWD cells can be robust and cultured without osmoprotection [29]. Similar observations of robust CWD cells have been reported in other species, including even the initial observations of *Streptobacillus moniliformis* L-forms described by Klieneberger-Nobel [12, 34, 42, 46–49]. CWD bacteria have also been cultured in many diverse growth conditions, such as urine [50], rabbit caecal fluid [34] and human serum [40]. It was also demonstrated that CWD bacteria could be adapted to low osmolarity conditions [51]. These observations suggest that not all CWD bacteria are alike, and that they can be sub-grouped as osmotically sensitive variants that require specialized osmoprotective culture conditions for survival and more robust variants that do not.

Here, we review the historic literature and recent research into CWD bacterial transition, survival, proliferation and reversion. We present evidence that indicates that, at least in some circumstances, the CWD state is an alternate lifestyle that enables bacteria to live and even proliferate without a cell wall. We also discuss some of the implications of CWD bacteria in antimicrobial tolerance.

GENERATION OF CWD BACTERIA

There are many conditions that can generate CWD variants. These include exposure to cell wall degrading enzymes [44, 52–54]; heat stress [55]; cryogenic stress [56]; osmotic stress [57, 58]; nutrient limitation [55, 56, 59]; genotoxic stress [14]; antibodies and complement proteins [60, 61]; bacteriophages [61, 62]; compounds that inhibit cell wall biosynthesis [54, 63]; and mutation of genes involved in cell wall biosynthesis [24, 46, 64, 65]. In this section, we will address whether the method by which a CWD state is achieved results in differences in the viability, robustness and physiology of the resulting CWD variant. We will mainly focus on antibiotic and enzymatic induction of CWD cells, as the mechanisms of bacterial cell wall changes in response to environmental stressors have recently been detailed elsewhere [66]. Through examination of the literature describing the aforementioned processes of generating CWD bacteria, there are two general mechanisms by which CWD could be derived: inhibition, mutation or substrate depletion of the enzymes required for cell wall biosynthesis; or enzymatic degradation or mechanical disruption of the cell wall.

Cell wall targeting antibiotics

There are many excellent reviews on cell wall synthesis in bacteria and detailed discussion of these processes is beyond the scope of this review; therefore, readers are directed to the recent review by Kumar *et al.* [67] for a more thorough understanding. Briefly, a large number of enzymes are involved in the biosynthesis and remodelling of cell wall peptidoglycan [2]. In general, synthesis of the bacterial peptidoglycan sacculus occurs in three stages. Soluble precursors (UDP-*N*-acetylglucosamine and *N*-acetylmuramyl pentapeptide) are first synthesized in the cytoplasm. They are then linked to the transport lipid (undecaprenyl phosphate) to form the lipid-anchored disaccharide pentapeptide monomer subunit (lipid II). After flipping across the cytoplasmic membrane, the lipid component is released and the glycan chains are inserted into the growing peptidoglycan sacculus. Polymerization of the glycan chains occurs through the activity of glycosyl transferases and peptide cross-linking occurs through the activity of DD-transpeptidases [2]. The DD-transpeptidases are also called penicillin binding proteins (PBPs) as they were first identified due to their affinity for binding the β-lactam antibiotic penicillin [68, 69].

From the very early days of antibiotic research, it was observed that antibiotics that target key steps in bacterial cell wall biosynthesis produce CWD variants in many species of bacteria [7, 15, 17, 25, 29, 45, 70, 71]. The β-lactam class of antibiotics includes penicillins, cephalosporins, carbapenems and monobactams. All β-lactam antibiotics possess a highly reactive 3-carbon, 1 nitrogen (β-lactam) ring. They function by binding to and acylating PBPs, thereby inhibiting the final transpeptidase steps of peptidoglycan biosynthesis, often leading to cell lysis [72].

The morphological response to different β-lactam antibiotics appears to be determined by the PBP binding affinity [15, 73, 74]. For example, in *E. coli*, the cephalosporin cefsulodin has highest affinity for PBP1a/1b and the penicillin amdinocillin (also known as mecillinam) binds almost exclusively to PBP2. Treatment with either of these antibiotics resulted in CWD cells, whereas treatment with mezlocillin, another penicillin-class antibiotic with an almost exclusive affinity for PBP3, resulted in long filamentous cells [74–76]. These observations were not limited to *E. coli*. Similar results were observed with mecillinam (amdinocillin) treatment of *Pseudomonas aeruginosa*, *Klebsiella aerogenes*, *Proteus rettgeri* and *Enterobacter cloacae* [77]. Treatment of *Pseudomonas aeruginosa* with the PBP2-binding carbapenem β-lactam antibiotic imipenem resulted in a conversion to round cells within 2 h, whereas treatment with the PBP3-binding cephalosporin ceftazidime induced the formation of filaments [73, 78].

The concentration and duration of the antibiotic exposure also determines the morphological response of the cells in response to PBP binding by β-lactam antibiotics. In *E. coli*, treatment with ampicillin and penicillin G, which both have a high affinity for PBP2 and PBP3 and a slightly lower affinity for PBP1a/1b, resulted in bulging elongated cells at the lowest effective concentration but as the concentration increased, and PBP1a/1b became engaged, increasing proportions of CWD cells were observed [74]. Amoxicillin, another β-lactam antibiotic of the penicillin family, has a primary affinity towards PBP1a/1b and PBP2. *E. coli* treated with amoxicillin resulted in very little filamentation at a low concentration but as the concentration increased, and PBP3 binding occurred, a morphological response similar to treatment with ampicillin and penicillin G was observed [74].

In *E. coli*, inhibiting PBP1a/1b and PBP2 may result in similar gross morphology (i.e. round cells) but leads to different outcomes for the resulting CWD bacteria. Binding of PBP1a/1b by β-lactam antibiotics was found to result in spheroplasts that are fragile and sensitive to osmotic lysis, whereas binding of PBP2 resulted in osmotically stable and replicating round forms [74, 76]. The fragile CWD cells can still be propagated through careful media selection, notably the presence of divalent cations, which are thought to strengthen the OM in Gram-negative cells [19, 70, 79]. Microarray analysis of the response of *E. coli* to cefsulodin and mecillinam (amdinocillin) (PBP1a/1b and PBP2 inhibiting, respectively) identified striking differences in the cellular response to antibiotic challenge. Cefsulodin treatment had fewer effects on gene expression, whereas mecillinam (amdinocillin) treatment activated more genes, particularly those involved with stress responses and membrane remodelling [80]. The significance of these observations is discussed below.

Antibiotics that target earlier steps of the cell wall biosynthesis pathway have also been shown to induce robust CWD cells. The antibiotic fosfomycin inhibits the activity of MurA (UDP-*N*-acetylglucosamine-enolpyruvyltransferase), an enzyme that catalyses the first committed step of peptidoglycan biosynthesis [2, 81]. Fosfomycin was found in a drug screen to induce the formation of CWD cells in Gram-negative bacilli [82] and has been used to induce stable CWD cells in *E. coli*, *Staphylococcus aureus* and *Corynebacterium glutamicum* [19, 24, 83], and unstable CWD cells in *Proteus mirabilis* [84]. Targeting MraY, a translocase important in the formation of lipid I, with nucleoside antibiotics such as mureidomycin or tunicamycin, resulted in differing responses in Gram-negative and Gram-positive bacteria. Mureidomycin treatment of Gram-negative bacteria resulted in fragile spheroplasts, whereas robust CWD cells were observed when Gram-positive pathogens were treated with tunicamycin. These observations indicate that MraY inhibition leading to CWD conversion may cause species-specific responses [85–88]. D-Cycloserine inhibits alanine racemase (Alr) and D-alanine ligase (Ddl), two enzymes involved in the early stages of peptidoglycan precursor synthesis in the cytoplasm, and has been shown to induce robust CWD bacteria, alone or in combination with glycine, in a range of bacterial species [24, 89–92]. Therefore, it appears that CWD bacteria induced by antibiotics targeting steps earlier in the peptidoglycan biosynthetic pathway appear to be just as robust as those induced by PBP2-inhibiting β-lactam antibiotics, and that targeting PBP1a/1b specifically may result in more fragile CWD cells.

Other cell wall biosynthesis inhibitors

It is not just antibiotics that can disrupt steps in the cell wall biosynthetic pathway and lead to the formation of CWD bacteria. The amino acid glycine has also been shown to disrupt cell wall synthesis leading to CWD bacteria by substituting with L-alanine in peptidoglycan precursor molecules, with the resulting molecule being poorly utilized by downstream enzymes in the biosynthetic pathway [48, 93]. These glycine-induced CWD cells were found to be osmotically robust with reversion occurring upon removal of the inducer [48]. In a recent study, the monosaccharide L-arabinose was found to induce viable, transient *V. cholerae* CWD variants within 9 h [57]. Analysis of the morphological states during transition into the CWD state showed features characteristic of the transition observed in other Gram-negative bacteria treated with cell wall inhibiting β-lactam antibiotics such as meropenem [29, 33]. Genetic screens identified key components required for this response in *V. cholerae*. These indicated that L-arabinose substitutes into the galactose metabolic pathway, thereby blocking downstream metabolic processes and depleting the production of metabolites necessary for cell wall synthesis or inhibiting enzymes required for cell wall synthesis [57].

These observations indicate that not only is it direct targeting of enzymes in the cell wall biosynthetic pathway that can generate robust CWD variants, but also disruption at the very early stages of cell wall synthesis by glycine and L-arabinose can lead to robust CWD variants.

Other antibiotics

Antibiotics of other classes that do not directly target peptidoglycan biosynthesis have also been reported to produce cells with a spherical morphotype. Trimethoprim has been shown to induce spheroplasts in a dose-dependent manner in *Proteus vulgaris*, though not in *E. coli* or '*Vibrio percolans*' [63]. A similar antibiotic, aminopterin, caused the formation of 'swollen coccoidal' cells in *Serratia marcescens* [94]. Both antibiotics work by competitively blocking the biosynthesis of tetrahydrofolic acid (THA). THA is required for many necessary biological functions, including the synthesis of metabolites for the cell wall [95]. Interestingly, trimethoprim has also been shown to increase trehalose in *E. coli*, a sugar that is utilized by bacteria as an osmoprotectant, which could be beneficial for bacterial survival in the absence of a cell wall [96]. *E. coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* treated with protein synthesis blocking antibiotics (i.e. chloramphenicol, streptomycin, oxytetracycline, kanamycin or tobramycin) generated spheroplasts, similar to what was observed with amino acid starvation and heat treatment [97–99]. These observations suggest that generalized disruption of protein synthesis likely leads to depletion of proteins involved in cell wall synthesis and, therefore, defects in cell wall formation. As most studies have utilized electron microscope imaging to capture this phenomenon, little is known about the viability of these spheroplasts. More research is required to determine whether inhibiting protein synthesis by these antibiotics can lead to the formation of viable CWD bacteria or whether these spheroplasts are an unrecoverable step in the process to cell death.

Peptidoglycan-degrading enzymes

CWD variants can also be produced through the direct action of peptidoglycan-degrading enzymes such as lysozyme (also known as muramidase), a ubiquitous enzyme that hydrolyses the β-1,4-glycosidic bond in peptidoglycan [100, 101]. This enzyme has been used to degrade the cell wall in a range of Gram-positive species and mycobacteria resulting in the formation of viable spheroplasts and protoplasts [44, 52, 54]. In Gram-negative species, the addition of OM permeabilization molecules such as EDTA or lactoferrin is required to breach the OM and facilitate access of the enzyme to the peptidoglycan layer [21, 102–105]. Host-derived lytic enzymes can also induce CWD variants in the Gram-positive bacteria *Staphylococcus aureus* and *Bacillus subtilis* [53]. However, in that study, CWD cells were propagated in the presence of penicillin G, which raises the possibility that while lysozyme is necessary to convert the bacteria to a CWD state, antibiotic inhibition of the cell wall pathway is required for the bacteria to propagate in the CWD state.

In this section, we have identified the major mechanisms through which CWD variants can be generated. It seems that inhibition of PBP2 results in robust CWD cells that are resistant to osmotic lysis, while inhibition of PBP1a/1b creates fragile, osmotically sensitive CWD variants. However, inhibition of the PBPs is only one approach to inducing CWD variants. Antibiotics targeting enzymes early in the process of cell wall synthesis, such as fosfomycin and D-cycloserine, and non-antibiotics, such as glycine and L-arabinose, can also generate robust CWD variants. More research is needed to address whether these robust CWD variants are altered in other ways that are not apparent through examination of their morphology and response to osmotic and mechanical stress.

REQUIREMENTS FOR SURVIVAL AS A CWD VARIANT

Stable CWD variants are often created by successive passaging of the parental strain under inducing conditions, reducing the concentration of the inducing agent until it can be removed entirely and reversion of the CWD variant no longer occurs [106, 107]. Therefore, it is logical to expect that stable CWD strains occur due to mutations in genes essential for cell wall synthesis. Indeed, analysis of a stable CWD *E. coli* strain identified a truncated *mraY* gene that prohibited the cell from regenerating a cell wall [108]. However, genome sequencing of a stable CWD *L. monocytogenes* strain showed no consensus pattern of mutations that would explain the loss of the cell wall [107]. This indicates that the genetic alterations through which stable CWD strains are derived are complex and not currently well defined.

In the previous section, we described the various ways in which CWD bacteria could be generated and outlined which of those mechanisms resulted in robust CWD variants and which resulted in fragile CWD cells that required more fastidious environmental requirements to maintain viability. In this section, we will discuss how stable CWD variants are created, and what is required for CWD variants to remain viable in either a stable or transient state.

Role of the cell envelope

It has been thought that the cell wall is essential in maintaining bacterial robustness to physical stress, but in Gram-negative bacteria it is becoming increasingly clear that the OM also contributes to mechanical resilience in the presence and absence of a cell wall [5, 19, 40, 51]. In CWD *E. coli*, compromising the OM increased cell lysis following osmotic challenge, or treatment with the OM-targeting antibiotic polymyxin B, or disrupting LPS synthesis [5, 19]. A mutant defective in the OM protein LptD (involved in the assembly of LPS on the cell surface) failed to generate viable CWD cells. Mutants in other important OM proteins (encoded by *lpp*, *ompA* and *pal* sequences) had reduced OM stiffness and were more sensitive to lysis [5]. In *V. cholerae*, membrane alterations may also be regulated by the VxrAB regulatory system. In *V. cholerae* treated with the β-lactam penicillin

G, 102 genes/operons with a putative VxrB binding site were found to be upregulated, of which many were involved in functions related to the cell envelope [42]. This confirms earlier studies with a *V. cholerae* strain overexpressing a phosphomimetic VxrB ($VxrB^{D78E}$), which identified that 30% of all upregulated genes encoded proteins related to the cell envelope, a 1.5-fold increase in cell wall content, and a marked increase in survival following osmotic shock [109].

Antibiotic treatment can also induce OM alterations that would promote OM stiffness and, therefore, promote resilience in Gram-positive and Gram-negative CWD bacteria. Meropenem treatment of *A. baumannii* upregulated genes that fortified the OM and induced autolysins that may act to remodel the cell envelope [110]. In *Enterobacter cloacae*, the two-component system PhoPQ was found to be necessary for survival of meropenem-induced CWD cells over 24 h [111]. Further analysis of genes controlled by the PhoPQ regulon identified *arnT*, which encodes a transferase that adds aminoarabinose to lipid A in the OM [111]. This modification likely increases the OM stability and resilience to mechanical and osmotic pressure.

The Rcs stress response system has been shown to be upregulated in many Gram-negative bacteria treated with antibiotics that target the cell wall, such as penicillin G, cefsulodin and mecillinam (amdinocillin) [20, 80]. The Rcs system regulon is not fully understood, but it regulates many genes involved with colanic acid synthesis (part of the cellular polysaccharide capsule), osmoregulation, motility and stress responses [112, 113]. It is currently unclear what the role of colanic acid in CWD survival is. Many genes involved in colonic acid biosynthesis (*wcaE*, *galU*, *cpsB*, *gmd* and *wzxC*) were found to be upregulated in penicillin G-induced *E. coli* CWD cells [20]. This was not merely a consequence of upregulation of the Rcs regulon, as cells with transposon insertions in these genes failed to grow on penicillin G agar. This indicates that the capsule was essential for CWD survival [20]. Deletion of *cpsE*, a gene required for polysaccharide synthesis, also resulted in no viable CWD when plated onto cefsulodin agar [23].

These findings indicate that, at least in Gram-negative bacteria, antibiotics induce mechanisms to promote survival without a cell wall. Interestingly, penicillin treatment of the Gram-positive organism *Staphylococcus aureus* resulted in CWD cells with increased membrane stiffness compared to the walled state [27], indicating that alterations in inner membrane composition may also contribute to the viability of CWD cells.

Oxidative stress

β-Lactam antibiotics have been shown to induce intracellular hydrogen peroxide production and oxidative damage in bacteria [42, 114, 115]. Transcriptional analysis of a stable CWD *L. monocytogenes* strain, created through repeated passages with penicillin G on osmoprotective media, was performed in order to identify the requirements for a viable stable CWD strain [106]. This study identified 276 genes that were differentially regulated (>2.5-fold) in the stable CWD variant when compared to the parental strain, with the majority of genes being downregulated. Upregulated genes were involved in the stress response and many of the downregulated genes were in pathways associated with metabolism [106]. The overall transcriptomic profile points towards a response to increased osmotic and oxidative stress and cells entering into a dormant metabolic state.

It has been shown that deleting genes involved in cell wall synthesis was not sufficient to make a stable CWD strain. Deletion of the *murE* operon in *B. subtilis* resulted in CWD bacteria, but proliferation was limited and overrun by bacillary cells, which the authors attributed to spontaneous mutation in the xylose promotor of the conditional mutant [65]. This conditional mutant was then propagated by more conventional methods, which involved successively sub-culturing in high concentrations of penicillin G. Using this approach, a stable CWD variant was obtained. Comparing the sequence of this stable CWD variant to a *B. subtilis* reference strain and the parental strain identified one polymorphism in *yqiD*, which encodes a homologue of IspA in *E. coli*. IspA is an enzyme that catalyses the formation of farnesyl pyrophosphate in the isoprenoid pathway, a biochemical pathway that leads to the formation of several essential lipids involved in the synthesis of peptidoglycan and menaquinone, an essential component within the electron transport chain [65].

Contrary to expectations, it was the role of IspA in the menaquinone biosynthetic pathway, rather than in peptidoglycan synthesis, that was essential for producing this viable CWD variant. Further studies showed that a deletion downstream of *ispA* in the menaquinone biosynthetic pathway (*hepS*) was able to support a stable CWD strain, but a gene mutant later in the peptidoglycan synthetic pathway (*uppS*) did not [116]. It was subsequently discovered that the *ispA* deletion protected the CWD cell from oxidative damage, as CWD cells have high levels of reactive oxygen species (ROS). Indeed, it was found that *B. subtilis* cultured under anaerobic conditions negated the necessity for an *ispA* mutation, and mutation of genes that reduced the functionality of the electron transport chain (and, therefore, the production of ROS) promoted CWD survival [116]. It can be concluded, therefore, that ROS produced through aerobic respiration is lethal to CWD *B. subtilis* and mechanisms to protect against oxidative stress are essential. This is consistent with the upregulation of oxidative stress response genes in the expression profiles reported for the stable CWD *L. monocytogenes* variant [106]. The need to compensate for oxidative stress may also be species and strain dependent. In contrast to what was observed in *B. subtilis*, deletion of *ispA* is not needed for stable CWD growth in *E. coli*, *Staphylococcus aureus* or *C. glutamicum* induced into a CWD state through the use of the cell wall inhibiting drug fosfomycin [24].

Protection from oxidative stress is not only necessary for the survival of a stable CWD strain, but transient CWD bacteria also need mechanisms to cope with oxidative stress to survive in the CWD state. It has been reported that *E. coli* is readily able to transition into and out of the CWD state and proliferate in the CWD state when induced by fosfomycin or penicillin G and grown under anaerobic conditions, but that the cells died under aerobic conditions [19, 116]. However, aerobic growth of CWD *E. coli* could be achieved in the presence of a ROS scavenger [116]. It has also been observed that *E. coli* isolates induced to a transient CWD state with meropenem varied, with three of four isolates tested having an upregulation of ElaB, an inner membrane protein regulated by RpoS and associated with protection against oxidative stress [117]. The isolate without elevated ElaB was still resistant to oxidative stress, suggesting an alternate pathway is used to protect against oxidative stress in this strain [117]. This could indicate that these strains have alternative mechanisms to cope with oxidative stress and the loss of cell wall can be accommodated by species-specific and strain-specific stress responses. The requirement for protection from oxidative stress is also supported by several older studies on unstable CWD bacteria that showed anaerobic growth conditions supported CWD transition and growth [61, 118, 119]. Therefore, as with stable CWD variants, oxidative stress also affects survival of unstable CWD bacteria.

Managing the ROS produced during conversion is essential for the long-term survival of CWD cells. CWD bacteria induced through enzymatic or antibiotic treatment have been shown to upregulate genes that repress ROS production or repair oxidative damage, such as SOS and DNA repair mechanisms [20, 27, 42, 106]. In *E. coli*, overexpressed genes included those involved in responses to envelope stress, particularly the Rcs phosphorelay two-component system and genes regulated by this system such as colanic acid biosynthesis; DNA repair/SOS response; drug efflux and resistance; OM lipoproteins and LPS biosynthesis; iron homeostasis including the ferric uptake regulator (*fur*); sulfate assimilation and cysteine biosynthesis; and PBP1b (*mrcB*) [20]. These results confirm previous transcriptional data on CWD *E. coli* treated with mecillinam (amdinocillin) [80]. CWD variants of pathogenic *E. coli* strains similarly demonstrated upregulation of genes involved in oxidative stress responses and DNA repair/SOS response, and the CWD forms were shown to be resistant to oxidative stress [117]. RNA-seq analysis of *Staphylococcus aureus* CWD cells indicated genes involved in energy metabolism, stress responses, protein synthesis and virulence were upregulated [27]. Analysis of a *Staphylococcus aureus* mutant transposon library found genes involved with iron homeostasis, DNA repair, OM proteins and membrane biosynthesis were important for CWD survival [120].

While the overall expression profile may vary between Gram-negative and Gram-positive bacteria in the CWD state, upregulation of DNA repair mechanisms, altering membrane synthesis and modulating iron uptake appears to be a conserved response in the CWD state. Unstable *E. coli* CWD cells have been shown to have downregulated haem and iron transport mechanisms and increased expression of the Fur regulator, which acts to downregulate iron-acquisition mechanisms [20]. Transcriptomic analysis of meropenem-induced CWD *A. baumannii* found a decrease in expression of *ompW*, which encodes an OM protein required for iron acquisition and is part of the Fur regulon [110, 121]. Further evidence on the role of iron limitation in CWD bacteria was identified in a study on *V. cholerae*, which found that the VxrAB two-component signal transduction system controls its own expression and is upregulated in response to cell wall damage from β-lactam antibiotics, fosfomycin and D-cycloserine [109]. The regulation of cell wall synthesis genes by VxrAB only partially explained the tolerance of *V. cholerae* to penicillin G. The RNA profiles of a *V. cholerae* strain overexpressing $VxrB^{D78E}$ compared to a strain with an empty control vector identified 100 genes that were downregulated in the $VxrB^{D78E}$ strain, including genes involved with iron acquisition [109]. In *V. cholerae*, penicillin G induced intracellular hydrogen peroxide and expression of the Fur regulon, thereby limiting iron uptake to protect against free radical damage [42]. It appears, therefore, that a common response of Gram-negative bacteria to cell wall inhibition by antibiotics is to induce mechanisms to limit iron-based oxidative damage to aid survival in the absence of a cell wall.

The process by which the CWD state is induced may also play a role in how the CWD cell deals with oxidative stress. As mentioned in the previous section, cefsulodin has high affinity for PBP1a/1b and inhibition of these enzymes results in the formation of osmotically fragile spheroplasts, whereas mecillinam (amdinocillin), which has high affinity for PBP2, leads to the formation of robust CWD cells. Transcriptional data of *E. coli* treated with either cefsulodin, mecillinam (amdinocillin) or both antibiotics showed that many more genes were upregulated with mecillinam treatment [80]. Cefsulodin treatment induced some genes involved with general stress responses, whereas mecillinam treatment (either alone or in combination with cefsulodin) induced genes involved in stress response and iron acquisition, and the fumarate and nitrate reductase (FNR) regulon was highly upregulated [80]. This is consistent with a cell undergoing iron limitation and oxidative stress.

Differences in the gene expression profile in response to CWD conversion may also be species-specific. Transcriptional analysis of *Mycobacterium smegmatis*, *M. tuberculosis* and *Mycobacterium avium* subspecies *paratuberculosis* induced to a CWD state through a combined glycine and lysozyme treatment identified fold changes of gene expression >2 when compared to the uninduced parental strain of 8.4, 11.8 and 2.5% of genes, respectively [54]. Despite morphological similarity in the CWD conversion, the pattern of expression was remarkably different, particularly when comparing the non-pathogenic *M. smegmatis* to the two pathogenic *Mycobacterium* species. Both *M. tuberculosis* and *M. avium* subspecies *paratuberculosis* upregulated genes linked to envelope remodelling and DNA repair, similar to the responses of other CWD variants of other species [20, 54]. In comparison, *M. smegmatis* upregulated only a few genes, most of them of unknown function, as well as two bacterial peptidoglycan deacetylases that modify bacterial peptidoglycan to resist lysozyme degradation, and the ferric uptake regulator Fur (MSMEG_4487) [54]. As the other two mycobacterial pathogens are adapted to resist macrophage ROS mechanisms encountered during infection, these

strains may have other mechanisms of protecting against oxidative damage that *M. smegmatis* lacks, or those mechanisms are already highly expressed and so no change was observed when they transitioned into a CWD state. These results indicate that survival in the CWD state has different requirements depending on the ecological niche of the parental strain.

In summary, these observations indicate that the bacterial response to antibiotic-induced oxidative damage depends on the antibiotic target, and the response to oxidative stress in turn determines the fate of the CWD bacteria. In the absence of environmental factors to inhibit ROS production, the CWD inducing agent, therefore, has dual roles: block cell wall synthesis and/or promote membrane remodelling; and induce mechanisms to protect against associated ROS damage. Without both of these roles, such as would be encountered by targeted mutation of cell wall biosynthesis genes, a viable CWD is not formed and a fragile non-viable CWD cell is made.

REVERSION BACK TO A WALLED STATE

In this review, we have discussed the generation of CWD variants and the requirements to survive in a CWD state. In this section, we will look at the requirements for unstable CWD bacteria to revert back to a walled state when the inducing agent is removed, degraded or exhausted.

Before the prevalence of genomic and transcriptomic approaches, many studies examined how growth conditions affected reversion of CWD cells. In an early study, penicillin was used to induce CWD cells of Gram-positive and Gram-negative organisms, specifically *E. coli*, *Proteus mirabilis*, and isolates of *Enterococcus*, *Klebsiella*, coagulase-positive and coagulase-negative *Staphylococcus* and β-haemolytic *Streptococcus*, in both broth culture and on agar plates [122]. Removal of penicillin and the subsequent reversion to the walled state took, on average, 24 h in broth cultures and 3 days in agar plates for most strains except the *Staphylococcus* and *E. coli* isolates. However, recently, it has been shown that reversion can occur rapidly in *E. coli* and *Pseudomonas aeruginosa*, with peptidoglycan levels restored and cell shape restored within 4–6 h of antibiotic removal [19, 29, 41].

The rate of reversion is likely a consequence of the inducing condition. CWD *E. coli* treated with either cefsulodin, penicillin G or fosfomycin were examined for reversion following cessation of the antibiotic treatment. CWD cells induced by cefsulodin and penicillin G started to revert 6 h after antibiotic removal, but it took 9 h following removal of fosfomycin before rod-shaped cells appeared in the culture [19]. Fosfomycin targets a much earlier enzymatic step in the synthesis of the cell wall, so the longer delay in reversion may be due to depletion of precursor molecules. Interestingly, even with similar rates of reversion, CWD cells induced by cefsulodin or penicillin G displayed different morphologies during the reversion process. This may be as a consequence of the different target enzymes of these antibiotics and/or different gene expression profiles of the resulting CWD cells [19, 80].

The study of reversion in CWD bacteria has been used as a tool to examine the requirements for *de novo* morphogenesis of rod-shaped bacteria to understand how bacteria generate a non-spherical shape in the absence of peptidoglycan to act as a template. In *B. subtilis*, a stable CWD strain incapable of synthesizing peptidoglycan was able to regenerate a cell wall and resume a rod shape when the peptidoglycan synthesis genes were complemented back [123]. During normal walled growth, peptidoglycan synthesis can continue if either of PBP1a or PBP1b is disrupted in its activity. It was found that in *E. coli*, in the absence of any peptidoglycan template, only PBP1b can synthesize the cell wall and cause the reversion to a normal rod shape of lysozyme induced CWD cells [105, 124].

Peptidoglycan is not the only structural element that is needed for a CWD cell to return to its normal walled morphology. It was demonstrated in *E. coli* that the actin homologue MreB is required for the CWD cells to return to a bacillary morphology [41]. In cefsulodin-treated *E. coli*, the CWD population reverted back to the regular bacillary morphology following cessation of antibiotic treatment. However, when treated with A22, an antibiotic that inhibits MreB polymerization, the reverting cells were unable to recover normal morphology even though there was no defect in peptidoglycan synthesis [41].

Some OM proteins of Gram-negative bacteria have also been found to be essential in the reversion process. After lysozyme treatment, mutants of the *E. coli* lipoprotein Lpp were found to remain in a CWD state even in the absence of lysozyme [105]. Similarly, when β-lactam antibiotics were used to induce a CWD state under anaerobic conditions, *lpp, ompA* and *pal* mutants were defective in reverting back to a walled state on the removal of the antibiotic, with *lpp* essential for viable revertants [19, 105]. These proteins were also shown to be important in maintaining the membrane stiffness in CWD bacteria and resisting mechanical lysis. Lpp and Pal lipoproteins connect the OM and the peptidoglycan layers in *E. coli* and OmpA is important in OM stability [125–127]. These findings indicate that connecting the layers of the cell envelope and maintaining OM integrity are crucially important to the reversion of CWD bacteria.

As noted above, the Rcs system is necessary for CWD survival and has been shown to be upregulated in unstable *E. coli* CWD bacteria treated with cell wall inhibiting antibiotics [20, 23, 80]. The Rcs system also appears to be essential in the reversion to the bacillary state of CWD *E. coli* [105]. Strains lacking *rcsB*, *rcsC*, *rcsF* lysed by 2 h following removal of the inducing agent. Interestingly, an *rcsA* mutant was able to create viable revertants [105]. More research is needed to understand which genes in the Rcs regulon are essential in the reversion process.

The VxrAB two-component histidine kinase reporter system of *V. cholerae* has also been shown to be essential in the reversion process of CWD bacteria, as well as survival in the CWD state. *V. cholerae* produces viable unstable CWD cells in the presence of high concentrations of β-lactam antibiotics that can revert within hours back to a bacillary state following addition of a β-lactamase to degrade the antibiotic [109]. A wild-type *V. cholerae* strain treated with 20× the MIC of penicillin G, and then serially diluted and plated onto agar, showed no reduction in viable c.f.u. over 6 h of exposure; however, both Δ*vxrA* and Δ*vxrB* mutants showed a significant reduction in recovered c.f.u. [109]. Further investigations identified that a Δ*vxrAB* strain was able to transition to a CWD state upon treatment with penicillin G, but was unable to revert back to bacillary form following removal of the antibiotic [109]. This was also true when fosfomycin and L-arabinose were used to induce the CWD state, indicating that the role of VxrAB is universal for any agent targeting the cell wall [57, 109].

Many of the systems that are necessary for CWD to survive without a cell wall are also essential for CWD cells to revert to a walled state. Taken together, it can be seen that when the cell wall is removed, processes are set in motion that simultaneously allow for the CWD to survive and thrive without a cell wall and to revert to a walled state should environmental conditions change again.

PROLIFERATION IN THE ABSENCE OF A CELL WALL

CWD bacteria not only are able to transition and survive in a CWD state, but many are also capable of proliferating in the absence of a cell wall. Proliferation of stable and unstable CWD variants has been observed in a range of Gram-negative and Gram-positive bacteria [12, 24, 128–131]. A variety of mechanisms of proliferation in CWD variants have been described and can be grouped into four general mechanisms, which will be detailed in this section. We will also discuss processes that are essential for proliferation of CWD bacteria.

It is beyond the scope of this review to discuss the mechanisms of classical bacterial cytokinesis and readers are directed to an excellent review by Vedyaykin *et al.* [132]. Briefly, FtsZ is a widely conserved protein in prokaryotes which at an early stage of cell division locates to the mid-cell to form a Z-ring which begins the recruitment of many proteins associated with cell wall synthesis, remodelling and fission. The result of this process is the formation of two identical daughter cells. CWD cells by their very definition lack a functional cell wall, so replication strategies for CWD bacteria are distinct from classical bacterial cell division. Indeed, cytokinesis in CWD bacteria has been shown to be independent of FtsZ [65, 131]. FtsZ may be present but there is no discernible ring structure [79] or the sites of FtsZ accumulation are distinct from the site of cellular constriction [51]. In mycobacteria, *ftsZ* expression is downregulated in the CWD state [54] indicating the lack of need for this protein in proliferation while in a CWD state. Other proteins essential in cell structure and separation (MreB, MinD) [23, 131] and chromosome segregation (Soj, SpoOJ) have also been shown to be dispensable for proliferation of CWD bacteria [131].

Proliferation mechanisms described for CWD bacteria can be grouped into four main categories. One of these involves single-site and multiple-site constrictions of the cell body, leading to fragmentation of the cytoplasm in a process reminiscent of classic binary fission [128]. The second is extracellular budding through which smaller daughter cells are made from membrane blebs at the cell surface [117, 128]. The third process, known as extrusion and resolution, involves the formation of a protruding filament that bulges at points along the length after which the filament collapses and the bulges separate as progeny [65].

In the fourth mechanism, progeny are formed when the inner membrane invaginates to form vesicles or intracellular bodies. These are then released following lysis of the mother cell [128, 129]. These progeny cells are viable, metabolically active and can continue to grow and multiply [106, 107, 129, 130, 133, 134]. Early studies using light microscopy to observe proliferation of CWD forms of streptococci, staphylococci and *B. subtilis* referred to these mother cells as 'large bodies' and the intracellular progeny as 'viable granules' [133, 134]. Dienes observed that as long as the large bodies with intracellular granules were present, transfer to new cultures would allow growth of CWD cultures to continue, indicating that these contained viable progeny [134].

This fascinating replicative process was further explored in a series of elegant studies that utilized modern molecular biology and fluorescence microscopy techniques to explore the life-cycle of *L. monocytogenes* CWD bacteria [106, 107, 130]. It was found that stable CWD cells of *L. monocytogenes* are multi-nucleoid, containing on average a 10-fold increase in chromosome copies compared to normal cell wall cells [107]. Phospholipid accumulation along the inner membrane of the mother cell leads to the formation of internal progeny cells that accumulate most of the mother cell's cytoplasmic contents, including nucleic acids and proteins. The mother cell swells to accommodate this internal progeny until lysing, releasing the progeny cells that undergo membrane polarization and become metabolically active. This mechanism of proliferation has been described in many *Listeria* and *Enterococcus* species [107].

It has been observed that the same population of CWD bacteria can undergo many different types of proliferation [128]. The capacity to undergo different modes of proliferation may reflect the extent to which the CWD bacteria have retained structural elements of the cell wall and/or the ability to synthesize peptidoglycan [128, 135].

In a study of unstable *E. coli* CWD cells induced with cefsulodin, it was found that residual peptidoglycan remained following transition, even after 20 passages under CWD-inducing conditions. This residual peptidoglycan was essential for the proliferation

of the cells in the CWD state [19, 23]. This was confirmed using microscopic analysis of replicating unstable CWD *E. coli*, where replication was only seen to occur at sites of residual cell wall [41]. However, the requirement for peptidoglycan in CWD reproduction is not clear. The stable CWD *E. coli* stain LW1655F+ has been grown for many generations, shows no evidence of peptidoglycan and sequencing of genes involved in cell division has shown a defective *mraY* that encodes an enzyme essential in the formation of the lipid II precursor in peptidoglycan synthesis [108]. Reproduction in a CWD state has also been observed in *B. subtilis* mutants incapable of cell wall synthesis [123, 136]. In contrast, L-arabinose-induced CWD cells of *V. cholerae* were found to contain residual amounts of peptidoglycan but showed no evidence of proliferation, whereas cefsulodin-induced *E. coli* CWD were able to replicate through peptidoglycan-dependent mechanisms [23, 57]. These findings indicate that the presence of peptidoglycan in a CWD variant does not necessarily correlate with propensity for proliferation.

It appears that the method of proliferation may be dependent on the presence or absence of residual peptidoglycan. In an *E. coli* strain induced into an unstable CWD state through use of penicillin, division by single site constriction, multiple site constriction and budding appeared to utilize portions of the remaining cell wall and only occurred in the early rounds of proliferation following CWD conversion. In later rounds of proliferation, replication through internal membrane partition seemed to be the most common proliferation process [128]. Therefore, it is likely that when peptidoglycan fragments are present, replication is through mechanisms that require these peptidoglycan fragments, but in its absence other mechanisms are utilized.

Proliferation of CWD cells may be dependent on excess membrane synthesis. In *B. subtilis*, proliferation of lysozyme-induced protoplasts was dependent on overexpression of *accDA*, which encodes the carboxyltransferase subunit of acetyl-coenzyme A carboxylase [136]. It was determined that excessive AccDA increased cellular levels of malonyl-coenzyme A, which in turn is utilized by the fatty acid synthase type II enzyme system, leading to excessive production of phospholipids [136]. The authors postulate a model where excessive membrane production alters the surface area to volume ratio of the cell in the absence of a cell wall and leads to membrane invagination and subsequent resolution into multiple pleomorphic progeny. Whilst this increase in membrane production may account for why the cells formed cell-like partitions, it doesn't explain how the genetic information is copied to result in viable progeny. One explanation could be in how the study was conducted. In this study, *B. subtilis* was induced into a protoplast through lysozyme treatment and prevented from dividing by use of the FtsZ-inhibiting chemical benzamide [136]. In this circumstance, it is possible that chromosomal duplication has occurred even in the absence of cell segregation. When the cell wall was degraded through lysozyme treatment, the excess membrane could envelope the duplicated nuclear regions and then partition into pleomorphic daughter cells. More research is required to understand how genomes are copied and distributed during proliferation of CWD bacteria.

CWD bacterial replication may not be limited to only one mechanism at a time and may utilize multiple reproductive strategies simultaneously. Gumpert and Taubeneck identified multiple mechanisms of replication occurring in unstable *E. coli* CWD cells [128]. Studer *et al.* [130] observed in a stable *L. monocytogenes* CWD variant the spherical 'mother' CWD cell would develop an asymmetrical shape and protrude to form progeny cells in a process they described as budding-like. In other instances, they also observed that the mother CWD cell formed multiple evaginations that pinched off to the form the progeny cells that appeared to be still connected to the mother cell by a thin strand of membrane-like material [130]. Ramijan *et al.* [58] found that hyperosmotic stress induced filamentous actinomycetes (*Streptomyces* and *Kitasatospora*) to form CWD cells, which they referred to as S-cells. When the S-cells were grown for the duration of 7 days, the S-cells appeared under time-lapse microscopy to be proliferating where the parental 'mother cell' was seen to deform by vesiculation, blebbing or tubulation to release the progeny cells [58]. These observations suggest that CWD bacteria may not be limited to the use of one mechanism during proliferation.

CWD proliferation is a remarkable phenomenon and offers insight into alternative bacterial replication strategies. Determining whether differences in the mechanism of induction of the CWD state and/or species variation influences which CWD proliferation mechanisms are employed requires further research.

IMPLICATIONS OF TRANSIENT CWD BACTERIA IN ANTIBIOTIC TOLERANCE

CWD bacteria have been implicated in a range of diseases and have been identified in many clinical settings (comprehensively reviewed by Onwuamaegbu and colleagues [137]). In this section, we will discuss the involvement of CWD bacteria in chronic and recurrent infections, and how recent advances in our understanding of CWD bacteria may inform better treatment options.

CWD bacteria may play an important role in infections and recurrence or failure of antibiotic therapy. CWD variants from many species have been identified and correlated with febrile episodes in bone marrow recipients under high-antibiotic-dosage regimes [138]. The post-treatment recurrence of *Haemophilus influenzae* infections treated with penicillin has been attributed to conversion and reversion back from a CWD state [139]. In a recent report, unstable CWD *E. coli* were identified in the urine of patients with urinary tract infections who were being treated with antibiotics such as amoxicillin, cephalexin or fosfomycin [50]. Further investigation showed that a CWD *E. coli* isolate from a patient treated with fosfomycin was able to survive and proliferate in urine, and was able to revert back to a walled state when the cells were transferred to an osmoprotective medium without the presence of fosfomycin [50]. Therefore, it is likely that transition to a CWD form allows the bacteria to avoid antibiotic lysis and to persist and revert to a pathogenic state, and could be the cause of recurring persisting infections.

Reversible transitions into and out of a CWD state may confer tolerance to cell wall targeting antibiotics *in vivo*. Indeed, a number of *in vitro* studies have demonstrated this possibility. When clinical isolates from several Gram-negative species (except with the notable exception of *E. coli*) were treated with supra-MIC concentrations of meropenem, a subset of the bacterial populations tolerated the antibiotic exposure and converted into a CWD cell [26]. The CWD bacteria were able to rapidly revert back to their normal bacillary morphology when NDM-1 carbapenemase was added to the cultures to deactivate the meropenem [26]. Similar observations have been reported in many other important human pathogens, including *V. cholerae* and *Pseudomonas aeruginosa* [26, 29, 34]. Transition to a CWD state conferred tolerance of the antibiotic at many times higher than the reported MIC [26, 29]. These *en masse* morphological changes into and out of the CWD state are rapid and do not require mutation or acquisition of antimicrobial-resistance genes. It appears likely that reversible CWD transitions may be a mechanism of tolerance to β-lactam antibiotics that is inherent to many bacteria. The Gram-negative isolates were also shown to transition and survive in human sera [26], indicating that there may be a role for transition to the CWD state as an antibiotic-tolerance mechanism during infection.

The use of an antibiotic MIC alone to determine effectiveness and target dosing concentrations is increasingly coming under scrutiny [140, 141]. The observations that tolerance to supra-MIC antibiotic concentrations may occur through conversion to a CWD state provides further argument that the use of the MIC as the sole metric for antibiotic efficacy must be revisited. As discussed above, the robustness of CWD cells appears to be determined by which PBP is targeted and the antibiotic concentration. Therefore, targeting treatment options to induce inherently fragile CWD cells, which may be more susceptible to host clearance mechanisms, might be an important consideration as part of the therapeutic treatment strategy to overcome antibiotic tolerance. Indeed, we have observed that *Pseudomonas aeruginosa* CWD cells induced by meropenem have elevated sensitivity to antimicrobial peptides including LL-37 derived from human cathelicidin [29]. It is possible that cell wall deficiency may be a vulnerability that could be exploited to develop more effective antimicrobial therapeutics.

The transition to a CWD state may also serve as an intermediary step in the acquisition of antibiotic resistance. In β-lactam-induced CWD *Staphylococcus aureus*, the recovered cell-walled revertants maintained an elevated resistance to the inducing agent (penicillin G) through many generations [49]. The transient loss of cell wall integrity had led to the stable enhanced resistance through upregulation of PBP4 [49]. Enhanced resistance to antibiotics has also been reported in CWD bacteria isolated from rats infected with *M. tuberculosis*. Isolates recovered from the infected rat tissues showed increased resistance to ethambutol and streptomycin [142, 143]. Resistance to ethambutol, a cell wall inhibiting antibiotic, was to be expected; however, the resistance to streptomycin indicates that there may be a more global antibiotic-resistance mechanism occurring in CWD bacteria.

With the challenges of antibiotic resistance, phage therapy has been seen as a promising addition to the treatment options. However, from the very early days of research into CWD bacteria, bacteriophage treatment has been reported to induce CWD forms [61, 62]. A recent study found that *E. coli* strains that were exposed to meropenem to induce the formation of CWD cells were able to resist the action of bacteriophages [117]. Therefore, similar issues with CWD transition and reversion to escape phage attack could be expected. Caution should be taken with the use of phage therapies in combination with cell wall inhibiting antibiotics as one may render the other ineffective.

As discussed in this review, CWD variants vary greatly due to differences in induction methods. There are also species and strain differences that alter the fitness of the resulting CWD bacteria. A greater understanding of the types of CWD bacteria and their similarities and differences is essential to improve antibiotic effectiveness, prevent increased antimicrobial resistance and ultimately improve health outcomes.

CONCLUDING REMARKS

The ability to transition into, survive and even proliferate in a CWD state may be a widespread capability of many bacterial species, though it remains poorly understood. It appears that in many bacterial species, the transition to a viable CWD state is not simply a passive process that is an outcome of direct peptidoglycan degradation or inhibition of cell wall biosynthesis, but in some cases represents an adaptive lifestyle transition involving global changes in gene expression that results in the formation of robust CWD cells that can flourish in a specific environmental niche and tolerate cell wall damaging antibiotics. We expect that the application of modern research approaches aimed at understanding CWD bacteria will lead to a more comprehensive understanding of this fascinating alternate bacterial lifestyle. Furthermore, this knowledge will provide tools that can be deployed to determine when bacteria have switched to the CWD lifestyle in various environmental and clinical settings. This will be of particular importance in understanding the contribution of CWD variants to persistent and recurrent infections and the failure of antibiotic therapies, and may lead to novel therapeutic approaches to combat these infections.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

References

1. Young KD. The selective value of bacterial shape. *Microbiol Mol Biol Rev* 2006;70:660–703.
2. Typas A, Banzhaf M, Gross CA, Vollmer W. From the regulation of peptidoglycan synthesis to bacterial growth and morphology. *Nat Rev Microbiol* 2011;10:123–136.
3. Vollmer W, Blanot D, de Pedro MA. Peptidoglycan structure and architecture. *FEMS Microbiol Rev* 2008;32:149–167.
4. Auer GK, Weibel DB. Bacterial cell mechanics. *Biochemistry* 2017;56:3710–3724.
5. Rojas ER, Billings G, Odermatt PD, Auer GK, Zhu L, *et al*. The outer membrane is an essential load-bearing element in gram-negative bacteria. *Nature* 2018;559:617–621.
6. Domingue GJ, Woody HB. Bacterial persistence and expression of disease. *Clin Microbiol Rev* 1997;10:320–344.
7. DienesL, Weinberger HJ. The L forms of bacteria. *Bacteriol Rev* 1951;15:245–288.
8. Tabor CW. Stabilization of protoplasts and spheroplasts by spermine and other polyamines. *J Bacteriol* 1962;83:1101–1111.
9. Van Rensburg AJ. Properties of *Proteus mirabilis* and providence spheroplasts. *J Gen Microbiol* 1969;56:257–264.
10. Schuhardt VT, Klesius PH. Osmotic fragility and viability of lysostaphin-induced staphylococcal spheroplasts. *J Bacteriol* 1968;96:734–737.
11. Perty M. Zur kenntnis kleinster Lebensformen nach Bau, Funktionen, Systemtik mit Spezialverzeichniss der in der Schweiz beobachteten. *Mittheilungen der Naturforschenden Gesellschaft in Bern* 1852:231–232.
12. Klieneberger E. The natural occurrence of pleuropneumonia-like organism in apparent symbiosis with *Streptobacillus moniliformis* and other bacteria. *J Pathol* 1935;40:93–105.
13. Dienes L. Organisms of Klieneberger and *Streptobacillus moniliformis*. *J Infect Dis* 1939;65:24–42. ;
14. Zou J, Kou S-H, Xie R, VanNieuwenhze MS, Qu J, *et al*. Non-walled spherical *Acinetobacter baumannii* is an important type of persister upon β-lactam antibiotic treatment. *Emerg Microbes Infect* 2020;9:1149–1159.
15. Bernabeu-Wittel M, García-Curiel A, Pichardo C, Pachón-Ibáñez ME, Jiménez-Mejías ME, *et al*. Morphological changes induced by imipenem and meropenem at sub-inhibitory concentrations in *Acinetobacter baumannii*. *Clin Microbiol Infect* 2004;10:931–934.
16. Hanberger H, Nilsson LE, Nilsson M, Maller R. Post-antibiotic effect of beta-lactam antibiotics on gram-negative bacteria in relation to morphology, initial killing and MIC. *Eur J Clin Microbiol Infect Dis* 1991;10:927–934.
17. GebickiJM, James AM. The preparation and properties of spheroplasts of *Aerobacter aerogenes*. *J Gen Microbiol* 1960;23:9–18.
18. Horii T, Kobayashi M, Sato K, Ichiyama S, Ohta M. An in-vitro study of carbapenem-induced morphological changes and endotoxin release in clinical isolates of gram-negative bacilli. *J Antimicrob Chemother* 1998;41:435–442.
19. Chikada T, Kanai T, Hayashi M, Kasai T, Oshima T, *et al*. Direct observation of conversion from walled cells to wall-deficient L-form and v*ice versa* in *Escherichia coli* indicates the essentiality of the outer membrane for proliferation of L-form cells. *Front Microbiol* 2021;12:645965.
20. Glover WA, Yang Y, Zhang Y. Insights into the molecular basis of L-form formation and survival in *Escherichia coli*. *PLoS One* 2009;4:e7316.
21. Sun Y, Sun T-L, Huang HW. Physical properties of *Escherichia coli* spheroplast membranes. *Biophys J* 2014;107:2082–2090.
22. Liu I, Liu M, Shergill K. The effect of spheroplast formation on the transformation efficiency in *Escherichia coli* DH5α. *J Exp Microbiol Immunol* 2006;9:81–85.
23. Joseleau-Petit D, Liébart J-C, Ayala JA, D'Ari R. Unstable *Escherichia coli* L forms revisited: growth requires peptidoglycan synthesis. *J Bacteriol* 2007;189:6512–6520.
24. Mercier R, Kawai Y, Errington J. General principles for the formation and proliferation of a wall-free (L-form) state in bacteria. *Elife* 2014;3:e04629.
25. Nakao M, Nishi T, Tsuchiya K. In vitro and in vivo morphological response of *Klebsiella pneumoniae* to cefotiam and cefazolin. *Antimicrob Agents Chemother* 1981;19:901–910.
26. Cross T, Ransegnola B, Shin J-H, Weaver A, Fauntleroy K, *et al*. Spheroplast-mediated carbapenem tolerance in Gram-negative pathogens. *Antimicrob Agents Chemother* 2019;63:e00756-19.
27. Xu Y, Zhang B, Wang L, Jing T, Chen J, *et al*. Unusual features and molecular pathways of *Staphylococcus aureus* L-form bacteria. *Microb Pathog* 2020;140:103970.
28. Ratnam S, Chandrasekhar S. The pathogenicity of spheroplasts of *Mycobacterium tuberculosis*. *Am Rev Respir Dis* 1976;114:549–554.
29. Monahan LG, Turnbull L, Osvath SR, Birch D, Charles IG, *et al*. Rapid conversion of *Pseudomonas aeruginosa* to a spherical cell morphotype facilitates tolerance to carbapenems and penicillins but increases susceptibility to antimicrobial peptides. *Antimicrob Agents Chemother* 2014;58:1956–1962.
30. Watanakunakorn C, Hamburger M. Induction of spheroplasts of *Pseudomonas aeruginosa* by carbenicillin. *Appl Microbiol* 1969;17:935–937.
31. Yang C, Li H, Zhang T, Chu Y, Zuo J, *et al*. Study on antibiotic susceptibility of *Salmonella typhimurium* L forms to the third and forth generation cephalosporins. *Sci Rep* 2020;10:3042.
32. Michailova L, Kussovsky V, Radoucheva T, Jordanova M, Markova N. Persistence of *Staphylococcus aureus* L-form during experimental lung infection in rats. *FEMS Microbiol Lett* 2007;268:88–97.
33. Weaver AI, Murphy SG, Umans BD, Tallavajhala S, Onyekwere I, *et al*. Genetic determinants of penicillin tolerance in *Vibrio cholerae*. *Antimicrob Agents Chemother* 2018;62:e01326-18.
34. Dörr T, Davis BM, Waldor MK. Endopeptidase-mediated beta lactam tolerance. *PLoS Pathog* 2015;11:e1004850.
35. Allan EJ, Hoischen C, Gumpert J. Bacterial L-forms. *Adv Appl Microbiol* 2009;68:1–39.
36. Rogers MJ, Simmons J, Walker RT, Weisburg WG, Woese CR, *et al*. Construction of the mycoplasma evolutionary tree from 5S rRNA sequence data. *Proc Natl Acad Sci USA* 1985;82:1160–1164.
37. Chen L-L, Chung W-C, Lin C-P, Kuo C-H. Comparative analysis of gene content evolution in phytoplasmas and mycoplasmas. *PLoS One* 2012;7:e34407.
38. Errington J, Mickiewicz K, Kawai Y, Wu LJ. L-form bacteria, chronic diseases and the origins of life. *Philos Trans R Soc Lond B Biol Sci* 2016;371:20150494.
39. Casadesús J. Bacterial L-forms require peptidoglycan synthesis for cell division. *Bioessays* 2007;29:1189–1191.

40. Cross T, Ransegnola B, Shin J-H, Weaver A, Fauntleroy K, *et al*. Spheroplast-mediated carbapenem tolerance in gram-negative pathogens. *Antimicrob Agents Chemother* 2019;63:e00756-19.
41. Billings G, Ouzounov N, Ursell T, Desmarais SM, Shaevitz J, *et al*. De novo morphogenesis in L-forms via geometric control of cell growth. *Mol Microbiol* 2014;93:883–896.
42. Shin J-H, Choe D, Ransegnola B, Hong H-R, Onyekwere I, *et al*. A multifaceted cellular damage repair and prevention pathway promotes high-level tolerance to β-lactam antibiotics. *EMBO Rep* 2021;22:e51790.
43. Panos C, Barkulis SS, Streptococcal L forms I. Effect of osmotic change on viability. *J Bacteriol* 1959;78:247–252.
44. Weibull C. The isolation of protoplasts from *Bacillus megaterium* by controlled treatment with lysozyme. *J Bacteriol* 1953;66:688–695.
45. Lederberg J. Bacterial protoplasts induced by penicillin. *Proc Natl Acad Sci USA* 1956;42:574–577.
46. de Pedro MA, Donachie WD, Höltje JV, Schwarz H. Constitutive septal murein synthesis in *Escherichia coli* with impaired activity of the morphogenetic proteins RodA and penicillin-binding protein 2. *J Bacteriol* 2001;183:4115–4126.
47. May JR, Roberts DE, Ingold A, Want SV. Osmotically stable L forms of *Haemophilus influenzae* and their significance in testing sensitivity to penicillins. *J Clin Pathol* 1974;27:560–564.
48. Want SV, May JR. Induction of L-forms of *Haemophilus influenzae* in vitro. *J Med Microbiol* 1975;8:369–373.
49. Fuller E, Elmer C, Nattress F, Ellis R, Horne G, *et al*. Beta-lactam resistance in *Staphylococcus aureus* cells that do not require a cell wall for integrity. *Antimicrob Agents Chemother* 2005;49:5075–5080.
50. Mickiewicz KM, Kawai Y, Drage L, Gomes MC, Davison F, *et al*. Possible role of L-form switching in recurrent urinary tract infection. *Nat Commun* 2019;10:4379.
51. Osawa M, Erickson HP. L form bacteria growth in low-osmolality medium. *Microbiology* 2019;165:842–851.
52. King JR, Gooder H. Induction of enterococcal L-forms by the action of lysozyme. *J Bacteriol* 1970;103:686–691.
53. Kawai Y, Mickiewicz K, Errington J. Lysozyme counteracts β-lactam antibiotics by promoting the emergence of L-form bacteria. *Cell* 2018;172:1038–1049.
54. Rosu V, Bandino E, Cossu A. Unraveling the transcriptional regulatory networks associated to mycobacterial cell wall defective form induction by glycine and lysozyme treatment. *Microbiol Res* 2013;168:153–164.
55. Markova N, Slavchev G, Michailova L, Jourdanova M. Survival of *Escherichia coli* under lethal heat stress by L-form conversion. *Int J Biol Sci* 2010;6:303–315.
56. Slavchev G, Michailova L, Markova N. Stress-induced L-forms of *Mycobacterium bovis*: a challenge to survivability. *New Microbiol* 2013;36:157–166.
57. Espinosa E, Daniel S, Hernández SB, Goudin A, Cava F, *et al*. L-Arabinose induces the formation of viable non-proliferating spheroplasts in *Vibrio cholerae*. *Appl Environ Microbiol* 2020;87:e02305-20.
58. Ramijan K, Ultee E, Willemse J, Zhang Z, Wondergem JAJ, *et al*. Stress-induced formation of cell wall-deficient cells in filamentous actinomycetes. *Nat Commun* 2018;9:5164.
59. Mearls EB, Izquierdo JA, Lynd LR. Formation and characterization of non-growth states in *Clostridium thermocellum*: spores and L-forms. *BMC Microbiol* 2012;12:180.
60. McIntosh D, Austin B. Recovery of cell wall deficient forms (L-forms) of the fish pathogens *Aeromonas salmonicida* and *Yersinia ruckeri*. *Syst Appl Microbiol* 1990;13:378–381.
61. DienesL, WeinbergerHJ, Madoff S. The transformation of typhoid bacilli into L forms under various conditions. *J Bacteriol* 1950;59:755–764.
62. Ongenae V, Mabrouk AS, Crooijmans M, Rozen D, Briegel A, *et al*. Reversible bacteriophage resistance by shedding the bacterial cell wall. *Open Biol* 2021;12:210379.
63. Dulaney EL, Marx LM. A folic acid linked system in bacterial cell wall synthesis? *J Antibiot* 1971;24:713–714.
64. Domínguez-Cuevas P, Mercier R, Leaver M, Kawai Y, Errington J. The rod to L-form transition of *Bacillus subtilis* is limited by a requirement for the protoplast to escape from the cell wall sacculus. *Mol Microbiol* 2012;83:52–66.
65. Leaver M, Domínguez-Cuevas P, Coxhead JM, Daniel RA, Errington J. Life without a wall or division machine in *Bacillus subtilis*. *Nature* 2009;457:849–853.
66. Mueller EA, Levin PA. Bacterial cell wall quality control during environmental tress. *mBio* 2020;11:e02456-20.
67. Kumar S, Mollo A, Kahne D, Ruiz N. The bacterial cell wall: from lipid II flipping to polymerization. *Chem Rev* 2022;122:8884–8910.
68. Sauvage E, Kerff F, Terrak M, Ayala JA, Charlier P. The penicillin-binding proteins: structure and role in peptidoglycan biosynthesis. *FEMS Microbiol Rev* 2008;32:234–258.
69. Macheboeuf P, Contreras-Martel C, Job V, Dideberg O, Dessen A. Penicillin binding proteins: key players in bacterial cell cycle and drug resistance processes. *FEMS Microbiol Rev* 2006;30:673–691.
70. LederbergJ, St Clair J. Protoplasts and L-type growth of *Escherichia coli*. *J Bacteriol* 1958;75:143–160.
71. Chang TW, Weinstein L. Morphological changes in gram-negative bacilli exposed to cephalothin. *J Bacteriol* 1964;88:1790–1797.
72. Pandey N, Cascella M. *Beta Lactam Antibiotics*. Treasure Island, FL: StatPearls Publishing; 2022.
73. Jackson JJ, Kropp H. Differences in mode of action of β-lactam antibiotics influence morphology, LPS release and in vivo antibiotic efficacy. *J Endotoxin Res* 2016;3:201–218.
74. Curtis NA, Orr D, Ross GW, Boulton MG. Affinities of penicillins and cephalosporins for the penicillin-binding proteins of *Escherichia coli* K-12 and their antibacterial activity. *Antimicrob Agents Chemother* 1979;16:533–539.
75. Spratt BG. Properties of the penicillin-binding proteins of *Escherichia coli* K12. *Eur J Biochem* 1977;72:341–352.
76. Spratt BG. Distinct penicillin binding proteins involved in the division, elongation, and shape of *Escherichia coli* K12. *Proc Natl Acad Sci USA* 1975;72:2999–3003.
77. Curtis NA, Orr D, Ross GW, Boulton MG. Competition of beta-lactam antibiotics for the penicillin-binding proteins of *Pseudomonas aeruginosa*, *Enterobacter cloacae*, *Klebsiella aerogenes*, *Proteus rettgeri*, and *Escherichia coli*: comparison with antibacterial activity and effects upon bacterial morphology. *Antimicrob Agents Chemother* 1979;16:325–328.
78. Sumita Y, Fukasawa M, Okuda T. Comparison of two carbapenems, SM-7338 and imipenem: affinities for penicillin-binding proteins and morphological changes. *J Antibiot* 1990;43:314–320.
79. Onoda T, Enokizono J, Kaya H, Oshima A, Freestone P, *et al*. Effects of calcium and calcium chelators on growth and morphology of *Escherichia coli* L-form NC-7. *J Bacteriol* 2000;182:1419–1422.
80. Laubacher ME, Ades SE. The Rcs phosphorelay is a cell envelope stress response activated by peptidoglycan stress and contributes to intrinsic antibiotic resistance. *J Bacteriol* 2008;190:2065–2074.
81. Silver LL. Fosfomycin: mechanism and resistance. *Cold Spring Harb Perspect Med* 2017;7:a025262.
82. Rodicio MR, Manzanal MB, Hardisson C. Protoplast-like structures formation from two species of *Enterobacteriaceae* by fosfomycin treatment. *Arch Microbiol* 1978;118:219–221.
83. Schmid EN. Fosfomycin-induced protoplasts and L-forms of *Staphylococcus aureus*. *Chemotherapy* 1984;30:35–39.
84. Schmid EN. Unstable L-form of *Proteus mirabilis* induced by fosfomycin. *Chemotherapy* 1985;31:286–291.

85. Isono F, Katayama T, Inukai M, Haneishi T. Mureidomycins A-D, novel peptidylnucleoside antibiotics with spheroplast forming activity. III. Biological properties. *J Antibiot* 1989;42:674–679.
86. Isono F, Inukai M, Takahashi S, Haneishi T, Kinoshita T, *et al*. Mureidomycins A-D, novel peptidylnucleoside antibiotics with spheroplast forming activity. II. Structural elucidation. *J Antibiot* 1989;42:667–673.
87. Isono F, Inukai M. Mureidomycin A, a new inhibitor of bacterial peptidoglycan synthesis. *Antimicrob Agents Chemother* 1991;35:234–236.
88. Zhu X, Liu D, Singh AK, Drolia R, Bai X, *et al*. Tunicamycin mediated inhibition of wall teichoic acid affects *Staphylococcus aureus* and *Listeria monocytogenes* cell morphology, biofilm formation and virulence. *Front Microbiol* 2018;9:1352.
89. Michel MF, Hijmans W. The additive effect of glycine and other amino acids on the induction of the L-phase of group A -haemolytic streptococci by penicillin and D-cycloserine. *J Gen Microbiol* 1960;23:35–46.
90. DienesL, Zamecnik PC. Transformation of bacteria into L forms by amino acids. *J Bacteriol* 1952;64:770–771.
91. Watanakunakorn C. Cycloserine induction, propagation, and antimicrobial susceptibility of wall-defective *Staphylococcus aureus*. *Infect Immun* 1971;3:438–443.
92. Azam MA, Jayaram U. Inhibitors of alanine racemase enzyme: a review. *J Enzyme Inhib Med Chem* 2016;31:517–526.
93. Hammes W, Schleifer KH, Kandler O. Mode of action of glycine on the biosynthesis of peptidoglycan. *J Bacteriol* 1973;116:1029–1053.
94. NickersonWJ, Webb M. Effect of folic acid analogues on growth and cell division of nonexacting microorganisms. *J Bacteriol* 1956;71:129–139.
95. Bermingham A, Derrick JP. The folic acid biosynthesis pathway in bacteria: evaluation of potential for antibacterial drug discovery. *Bioessays* 2002;24:637–648.
96. AlRabiah H, Allwood JW, Correa E, Xu Y, Goodacre R. pH plays a role in the mode of action of trimethoprim on *Escherichia coli*. *PLoS One* 2018;13:e0200272.
97. Klainer AS, Russell RR. Effect of the inhibition of protein synthesis on the *Escherichia coli* cell envelope. *Antimicrob Agents Chemother* 1974;6:216–224.
98. Klainer AS, Perkins RL. Surface manifestations of antibiotic-induced alterations in protein synthesis in bacterial cells. *Antimicrob Agents Chemother* 1972;1:164–170.
99. Waisbren SJ, Hurley DJ, Waisbren BA. Morphological expressions of antibiotic synergism against *Pseudomonas aeruginosa* as observed by scanning electron microscopy. *Antimicrob Agents Chemother* 1980;18:969–975.
100. Salton MR. The properties of lysozyme and its action on microorganisms. *Bacteriol Rev* 1957;21:82–100.
101. SaltonMR, Ghuysen JM. The structure of di- and tetrasaccharides released from cell walls by lysozyme and *Streptomyces* F 1 enzyme and the beta(1 to 4) N-acetylhexos-aminidase activity of these enzymes. *Biochim Biophys Acta* 1959;36:552–554.
102. Birdsell DC, Cota-Robles EH. Production and ultrastructure of lysozyme and ethylenediaminetetraacetate-lysozyme spheroplasts of *Escherichia coli*. *J Bacteriol* 1967;93:427–437.
103. Marvin HJ, Witholt B. A highly efficient procedure for the quantitative formation of intact and viable lysozyme spheroplasts from *Escherichia coli*. *Anal Biochem* 1987;164:320–330.
104. Ellison RT, Giehl TJ. Killing of gram-negative bacteria by lactoferrin and lysozyme. *J Clin Invest* 1991;88:1080–1091.
105. Ranjit DK, Young KD. The Rcs stress response and accessory envelope proteins are required for de novo generation of cell shape in *Escherichia coli*. *J Bacteriol* 2013;195:2452–2462.
106. Dell'Era S, Buchrieser C, Couvé E, Schnell B, Briers Y, *et al*. *Listeria monocytogenes* L-forms respond to cell wall deficiency by modifying gene expression and the mode of division. *Mol Microbiol* 2009;73:306–322.
107. Briers Y, Staubli T, Schmid MC, Wagner M, Schuppler M, *et al*. Intracellular vesicles as reproduction elements in cell wall-deficient L-form bacteria. *PLoS One* 2012;7:e38514.
108. Siddiqui RA, Hoischen C, Holst O, Heinze I, Schlott B, *et al*. The analysis of cell division and cell wall synthesis genes reveals mutationally inactivated ftsQ and mraY in a protoplast-type L-form of *Escherichia coli*. *FEMS Microbiol Lett* 2006;258:305–311.
109. Dörr T, Alvarez L, Delgado F, Davis BM, Cava F, *et al*. A cell wall damage response mediated by a sensor kinase/response regulator pair enables beta-lactam tolerance. *Proc Natl Acad Sci USA* 2016;113:404–409.
110. Islam N, Kazi MI, Kang KN, Biboy J, Gray J, *et al*. Peptidoglycan recycling promotes outer membrane integrity and carbapenem tolerance in *Acinetobacter baumannii*. *mBio* 2022;13:e01001-22.
111. Murtha AN, Kazi MI, Schargel RD, Cross T, Fihn C, *et al*. High-level carbapenem tolerance requires antibiotic-induced outer membrane modifications. *PLoS Pathog* 2022;18:e1010307.
112. Guo X-P, Sun Y-C. New insights into the non-orthodox two component Rcs phosphorelay system. *Front Microbiol* 2017;8:2014.
113. Majdalani N, Gottesman S. The Rcs phosphorelay: a complex signal transduction system. *Annu Rev Microbiol* 2005;59:379–405.
114. Van Acker H, Coenye T. The role of reactive oxygen species in antibiotic-mediated killing of bacteria. *Trends Microbiol* 2017;25:456–466.
115. Zhao X, Drlica K. Reactive oxygen species and the bacterial response to lethal stress. *Curr Opin Microbiol* 2014;21:1–6.
116. Kawai Y, Mercier R, Wu LJ, Domínguez-Cuevas P, Oshima T, *et al*. Cell growth of wall-free L-form bacteria is limited by oxidative damage. *Curr Biol* 2015;25:1613–1618.
117. Fabijan AP, Kamruzzaman M, Martinez-Martin D, Venturini C, Mickiewicz K, *et al*. L-form switching confers antibiotic, phage and stress tolerance in pathogenic *Escherichia coli*. *bioRxiv* 2021:449206.
118. Huber TW, Brinkley AW. Growth of cell wall-defective variants of *Escherichia coli*: comparison of aerobic and anaerobic induction frequencies. *J Clin Microbiol* 1977;6:166–171.
119. Seeberg S. Induction and surface growth of l-phase variants of different *Escherichia coli* strains. *Acta Pathol Microbiol Scand B Microbiol Immunol* 2009;81B:703–706. ;
120. Han J, Shi W, Xu X, Wang S, Zhang S, *et al*. Conditions and mutations affecting *Staphylococcus aureus* L-form formation. *Microbiology* 2015;161:57–66.
121. Nwugo CC, Gaddy JA, Zimbler DL, Actis LA. Deciphering the iron response in *Acinetobacter baumannii*: proteomics approach. *J Proteomics* 2011;74:44–58.
122. Nimmo LN, Blazevic DJ. Selection of media for the isolation of common bacterial L-phase organisms from a clinical specimen. *Appl Microbiol* 1969;18:535–541.
123. Kawai Y, Mercier R, Errington J. Bacterial cell morphogenesis does not require a preexisting template structure. *Curr Biol* 2014;24:863–867.
124. Ranjit DK, Jorgenson MA, Young KD. PBP1B glycosyltransferase and transpeptidase activities play different essential roles during the de novo regeneration of rod morphology in *Escherichia coli*. *J Bacteriol* 2017;199:e00612-16.
125. Wang Y. The function of OmpA in *Escherichia coli*. *Biochem Biophys Res Commun* 2002;292:396–401.
126. Szczepaniak J, Holmes P, Rajasekar K, Kaminska R, Samsudin F, *et al*. The lipoprotein Pal stabilises the bacterial outer membrane during constriction by a mobilisation-and-capture mechanism. *Nat Commun* 2020;11:1305.
127. Mathelié-Guinlet M, Asmar AT, Collet J-F, Dufrêne YF. *Lipoprotein* Lpp regulates the mechanical properties of the *E. coli* cell envelope. *Nat Commun* 2020;11:1789.
128. Gumpert J, Taubeneck U. Modes of multiplication in an unstable spheroplast type F-form of *Escherichia coli* K12(lambda). *Z Allg Mikrobiol* 1974;14:675 690–.

129. Green MT, Heidger PM, Domingue G. Proposed reproductive cycle for a relatively stable L-phase variant of *Streptococcus faecalis*. *Infect Immun* 1974;10:915–927.
130. Studer P, Staubli T, Wieser N, Wolf P, Schuppler M, *et al*. Proliferation of *Listeria monocytogenes* L-form cells by formation of internal and external vesicles. *Nat Commun* 2016;7:13631.
131. Mercier R, Domínguez-Cuevas P, Errington J. Crucial role for membrane fluidity in proliferation of primitive cells. *Cell Rep* 2012;1:417–423.
132. Vedyaykin AD, Ponomareva EV, Khodorkovskii MA, Borchsenius SN, Vishnyakov IE. Mechanisms of bacterial cell division. *Microbiol* 2019;88:245–260.
133. Burmeister HR, Hesseltine CW. Induction and propagation of a *Bacillus subtilis* L form in natural and synthetic media. *J Bacteriol* 1968;95:1857–1861.
134. Dienes L. Morphology and reproductive processes of the L forms of bacteria. I. Streptococci and straphylocci. *J Bacteriol* 1967;93:693–702.
135. Briers Y, Walde P, Schuppler M, Loessner MJ. How did bacterial ancestors reproduce? Lessons from L-form cells and giant lipid vesicles: multiplication similarities between lipid vesicles and L-form bacteria. *Bioessays* 2012;34:1078–1084.
136. Mercier R, Kawai Y, Errington J. Excess membrane synthesis drives a primitive mode of cell proliferation. *Cell* 2013;152:997–1007.
137. Onwuamaegbu ME, Belcher RA, Soare C. Cell wall-deficient bacteria as a cause of infections: a review of the clinical significance. *J Int Med Res* 2005;33:1–20.
138. Woo PC, Wong SS, Lum PN, Hui WT, Yuen KY. Cell-wall-deficient bacteria and culture-negative febrile episodes in bone-marrow-transplant recipients. *Lancet* 2001;357:675–679.
139. Lapinski EM, Flakas ED. Induction of L forms of *Haemophilus influenzae* in culture and their demonstration in human bronchial secretions. *J Bacteriol* 1967;93:1438–1445.
140. Landersdorfer CB, Nation RL. Limitations of antibiotic MIC-based PK-PD metrics: looking back to move forward. *Front Pharmacol* 2021;12:770518.
141. Wen X, Gehring R, Stallbaumer A, Riviere JE, Volkova VV. Limitations of MIC as sole metric of pharmacodynamic response across the range of antimicrobial susceptibilities within a single bacterial species. *Sci Rep* 2016;6:37907.
142. Markova N, Michailova L, Jourdanova M, Kussovski V, Valcheva V, *et al*. Exhibition of persistent and drug-tolerant L-form habit of *Mycobacterium tuberculosis* during infection in rats. *Open Life Sci* 2008;3:407–416.
143. Slavchev G, Michailova L, Markova N. L-form transformation phenomenon in *Mycobacterium tuberculosis* associated with drug tolerance to ethambutol. *Int J Mycobacteriol* 2016;5:454–459.