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Bacterial Metabolism and Susceptibility to Cell wall-active antibiotics

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Abstract

Bacterial infections are increasingly resistant to antimicrobial therapy. Intense research focus has thus been placed on identifying the mechanisms that bacteria use to resist killing or growth inhibition by antibiotics and the ways in which bacteria share these traits with one another. This work that has led to the advancement of new drugs, combination therapy regimens, and a deeper appreciation for the adaptability seen in microorganisms. However, while the primary mechanisms of action of most antibiotics are well-understood, the more subtle contributions of bacterial metabolic state to repairing or preventing damage caused by antimicrobials (thereby promoting survival) are still understudied. Here, we review a modern viewpoint on a classical system: examining bacterial metabolism's connection to antibiotic susceptibility. We dive into the relationship between metabolism and antibiotic efficiency through the lens of growth rate, energy state, resource allocation, and the infection environment, focusing on cell wall-active antibiotics.

Keywords

antibiotic resistance; antibiotic tolerance; beta-lactams

1. Introduction

The discovery of broad-spectrum antibiotics in the early 20th century revolutionized medicine and saved countless lives through both direct intervention (treatment of otherwise lethal infections) and through indirect effects, such as enabling organ transplants and chemotherapy via reduction of the probability of secondary infection (Baghdadi et al., 2021; Kaviani et al., 2020; Teillant et al., 2015). One of the first broadly used antibiotics was penicillin, of the β-lactam class (Bigger, 1944). β-lactams target the bacterial cell wall, and cell wall-active antibiotics have subsequently gained the status of some of our most powerful and most widely used antibiotic agents (Bush & Bradford, 2016). β-lactams are of particularly impressive clinical value due to their low toxicity to humans, combined with their typically bactericidal activity (Fietta et al., 1983). Cell wall-active antibiotics are generally quite potent, and the cell wall thus continues to be a prime target for the development of novel antimicrobial agents. Their unique value lies in their target – the bacterial cell wall, which, in most bacteria, is essential for growth, viability, and cell

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shape maintenance (Dörr et al., 2019). The cell wall consists mostly of peptidoglycan (PG), an aminosugar mesh where long polysaccharide strands are crosslinked with each other via short oligopeptide side stems. The polysaccharide strands are assembled from disaccharide-pentapeptide precursors ("lipid II", which is synthesized in the cytoplasm and then translocated to the outside via the permeases MurJ or AmJ) by the glycosyltransferase (GT) activities of class A PBPs (aPBPs, for short) or SEDS proteins (RodA and FtsW) (Cho et al., 2016; Dion et al., 2019; Garde et al., 2021; Lovering et al., 2012). Notably, lipid II requires the attached hydrophilic carrier molecule undecaprenol pyrophosphate (Und-PP) for translocation to the outside. Concomitant with and/or after GT activity, the polymerized precursor strands are then crosslinked with each other by the transpeptidase (TP) activity of class B PBPs (bPBPs), or the TP domains of aPBPs (Garde et al., 2021).

β-lactams target penicillin-binding proteins (PBPs), the primary cell wall synthases. More specifically, β-lactams inhibit the TP active site of PBPs, while not affecting GT activity (Malouin & Bryan, 1986; Spratt & Cromie, 1988). This is detrimental to the cell in surprisingly numerous ways: First, the resulting lack of crosslinking destabilizes the emerging PG network. In addition, however, the cell enters a nefarious cycle of production of long uncrosslinked PG strands followed by constant degradation by cell wall lytic enzymes, so-called autolysins, and this adds to the emerging lethal phenotype. In addition to this process, termed "futile cycling", autolysins (mostly endopeptidases and lytic transglycosylases) also directly contribute to the destruction of the existing PG sacculus, albeit in poorly understood ways (Cho et al., 2014; Dörr, 2021; Dörr et al., 2015; Kazuaki et al., 1986). The large-scale perturbations of cell wall homeostasis have other undesirable (from the bacterium's perspective) sequelae, including the generation of reactive oxygen species and reprogramming of central metabolism, which may also contribute to killing (Dwyer et al., 2014; Shin et al., 2021). Other cell-wall active antibiotics target the cell wall in different ways, e.g. through sequestration of lipid II, inhibition of cytosolic precursor synthesis, or GT inhibition (Fig. 1).

The primary mechanisms of action of most cell wall-active antibiotics are reasonably well-understood, and so are primary mechanisms of resistance. β-lactams, for example, are efficiently degraded by β-lactamases or rendered ineffective by target site mutation or production of alternative, β-lactam insensitive crosslinking enzymes (Tooke et al., 2019; Zapun et al., 2008). In addition to outright resistance, bacteria can also exhibit "tolerance", which is the ability to retain viability in the presence of antibiotics for extended time periods (Brauner et al., 2016; Dörr, 2021). The mechanisms of tolerance are less well understood, but typically rely on intrinsic damage repair or prevention pathways, rather than acquired resistance factors or mutations. Thus, both in the mechanism of killing and resistance to killing, there is considerable mechanistic grey area in our understanding of how CWAs attack bacteria, and how bacterial responses enable them to survive or even grow in the presence of CWAs. Indeed, in recent years, it has become increasingly appreciated that bacterial metabolic state significantly contributes to antibiotic susceptibility, in addition to the primary CWA effect of cell wall damage (Kawai et al., 2019; Liu et al., 2020; Zheng et al., 2020). However, the extent to which metabolic state truly contributes to antibiotic tolerance and resistance is still poorly understood. In this review, we will outline several key

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connections between bacterial physiology, antibiotic resistance, and tolerance, focusing on cell wall-active drugs.

2. Growth state and antibiotic susceptibility

Most cell wall-acting drugs only target actively growing cells. β-lactams, for example, bind and inhibit the transpeptidase activity of PBPs. This results in cell wall destruction by autolysins, which are most active during cellular elongation and rapid growth. It thus seems obvious that if growth is slowed or shut down, antibiotic tolerance to CWA is enhanced. Indeed, several studies, some of them dating back to the 1980s, firmly established the growth rate-dependency of β-lactam antibiotic efficacy as a contributor to tolerance (Horne & Tomasz, 1977; Tuomanen, 1986; Tuomanen et al., 1986), and implicated cell division as a particularly vulnerable point for lethal CWA activity (Chung et al., 2009; de Pedro et al., 2002). In the next section, we will discuss how metabolism, growth rate regulation, and tolerance to CWAs intersect.

2.1 Stringent Response: A major growth rate driver and tolerance determinant

Stress response systems contribute to a reduced growth rate, and the stringent response is a particularly well-understood example of this. Originally discovered in *E. coli* during amino acid starvation that inhibited protein synthesis (Cashel & Gallant, 1969), the stringent response is a complex global regulator of DNA replication, transcription, nucleotide synthesis, ribosome biogenesis, and function, as well as lipid metabolism (Irving et al., 2021). Due to its effect on a vast number of metabolic processes, the stringent response has strong and well-characterized effects on antibiotic susceptibility.

Cashel et al., discovered that pppGpp (guanosine 3′-diphosphate,5′-triphosphate) and ppGpp (guanosine 3′,5′-bispyrophosphate) accumulate in response to starvation (Gallant et al., 1971). In well-characterized model organisms, these nucleotides are synthesized by enzymatic phosphorylation of GDP and GTP to ppGpp and pppGpp, respectively, using ATP as a phosphate donor. The synthase, RelA, controls the levels of (p)ppGpp during amino acid starvation (Sinha & Winther, 2021), whereas the dual function enzyme SpoT acts as both a synthetase and a hydrolase that controls (p)ppGpp levels during carbon, iron, phosphate and fatty acid starvation (Germain et al., 2019). (p)ppGpp accumulation can be induced by diverse starvation conditions, including amino acid starvation, fatty acid starvation, and iron starvation. Many bacteria have alternative (p)ppGpp synthases and hydrolases, with some of them fulfilling specialized functions during cell envelope stress (Peyrusson et al., 2020).

Accumulation of (p)ppGpp results in massive changes to cellular physiology. In *E. coli*, (p)ppGpp binds directly to RNA polymerase at two separate sites (one of the sites in interaction with the cofactor DksA), resulting in large-scale alterations of global gene expression, including the reduction of transcription of stable RNAs (e.g., rRNAs) (Ross et al., 2016). In *B. subtilis,* the same end goal is achieved through ppGpp-mediated inhibition of GTP synthesis (Gourse et al., 2018). Since ribosome assembly is limited by rRNA levels, the stringent response induces a strong downregulation of the translation apparatus, concomitant with reduced growth. However, (p)ppGpp also positively controls transcription

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of a number of genes. Transcription of the translation apparatus genes and stable RNAs account for a large percentage (60-80%) of the transcription occurring in rapidly growing cells; the release of RNA polymerase from these genes is thought to passively promote upregulation of diverse promoters activated at the onset of stationary phase (Traxler et al., 2008), like other global stress response regulators (e.g., *rpoE*, *rpoH*, and *rpoS*) and their effectors.

The stringent response has been extensively studied in the context of its effect on the regulation of metabolism and energy state of the cell. For example, (p)ppGpp production during fatty acid starvation inhibits many metabolic activities, ensuring that the cell volume does not outstrip the cell envelope capacity, thus maintaining the integrity of the envelope (Vadia et al., 2017). (p)ppGpp regulates growth rate at multiple steps. For example, in *E. coli* and *B. subtilis* direct binding of (p)ppGpp to the active site of DnaG (the primase), downregulates DNA replication (Maciag et al., 2010). Indeed, DNA replication rate in *E.* coli is inversely correlated with ppGpp levels (Chiaramello & Zyskind, 1990), which should affect growth rate. (p)ppGpp also controls the expression of the DNA replication initiator ATPase, DnaA, and further affects DNA replication by indirectly controlling the binding of DnaA to *oriC* (Kraemer et al., 2019).

Levels of (p)ppGpp have also been implicated in the control of nucleotide synthesis, specifically purine synthesis (Kriel et al., 2012). In *E. coli*, ppGpp can inhibit PurF, which is responsible for the first step in the conversion of phosphoribosyl pyrophosphate (PRPP) to inosine monophosphate (IMP), thus halting *de novo* biosynthesis of all purine nucleotides. Two molecules of ppGpp bind to PurF at an allosteric site, disrupting the formation of the catalytic center and competitively inhibiting enzyme activity. (p)ppGpp can also inhibit the ATP biosynthesis enzyme PurA (Anderson et al., 2021). PurA catalyzes the committed step of AMP biosynthesis, and subsequent ATP generation, from IMP. GuaB is common to both the *de novo* and the salvage nucleotide synthesis pathways, and catalyzes the formation of xanthosine monophosphate from IMP, a reaction that is weakly inhibited by (p)ppGpp in *B. subtilis* (Kriel et al., 2012). Since PRPP is also a precursor molecule for Uridine Diphosphate (UDP), which plays a central role in cell wall and LPS synthesis as a carrier of monosaccharides, its effects on PurF may be a way by which the stringent response tunes cell envelope synthesis.

In principle, all of the (p)ppGpp-induced changes outlined above can affect growth rate, and thus CWA antibiotic susceptibility. For example, the stringent response has been linked to mecillinam resistance in *E.coli,* where ppGppp-controlled upregulation of FtsQAZ, likely coupled with slow growth, enables mecillinam-treated cells to divide as spherical shapes (Vinella et al., 2005). Stringent response activation has also been shown to promote CWA tolerance (suppression of lysis) in numerous studies in *E. coli*, largely through the effect of ppGpp on growth arrest; however, most studies were conducted via artificial upregulation of stringent response. Clearly, induction of the stringent response can increase tolerance in *E. coli*, but it is not clear if this is physiological (Corrigan et al., 2016; Das & Bhadra, 2020; Rodionov & Ishiguro, 1995). In *Pseudomonas aeruginosa*, however, inactivating the stringent responses suppresses CWA tolerance in biofilms (Nguyen et al., 2011). In *Staphyloccous aureus,* a ppGpp synthetase is under control of a cell wall stress sensing

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system, likewise implicating stringent response in protection from killing by CWAs (Geiger et al., 2014).

While the effect of the stringent response on growth-rate-related alterations of antibiotic susceptibility are intuitive, there are also more direct effects of ppGpp on antibiotic resistance. In *Helicobacter pylori,* for example, a glucose/galactose transporter, Hp1174, that functions as an efflux pump is highly expressed in biofilm-forming and multidrug-resistant (MDR) *H. pylori* strains. This transporter, encoded by the *gluP* gene, is upregulated by SpoT, likely in response to starvation (Ge et al., 2018).

As an intriguing side-note, one of the main targets of the stringent response, transcription, has another well-documented connection with cell envelope homeostasis and CWA susceptibility. Rifampicin-resistant point mutants in RNA polymerase often show pleiotropically altered susceptibility to CWAs (Patel et al., 2023), and sub-MIC rifampicin exposure prevents mecillinam resistance in *E. coli* (Vinella et al., 1992). Importantly, metabolomics showed that some Rif-resistant *rpoB* mutants in *B. subtilis* accumulated PG precursors, suggesting specific downregulation of cell wall synthesis functions in response to altered transcriptional activity.

In conclusion, (p)ppGpp levels modulate bacterial growth and thus have a profound effect on CWA susceptibility. However, shutting down bacterial growth alone does not necessarily shut down cell wall turnover, and indeed cell wall-active antibiotics can kill stationary phase bacteria (Eng et al., 1991; Tuomanen, 1986; Wu et al., 2018).

As described above, the earliest studies of the mechanism of action of β-lactam antibiotics established that these antibiotics only kill growing cells, and that starvation renders bacteria completely tolerant to these drugs. Several studies in the 1980s examined the physiological underpinnings of tolerance induced by growth arrest, using stringent response induction as the tolerance condition. Inducing the stringent response by, for example, inducing amino acid starvation, rendered *E. coli* completely resistant to killing by β-lactam antibiotics (Goodell & Tomasz, 1980; Tuomanen & Tomasz, 1986). Conversely, treatment of amino acid-deprived *relA* mutants (which are unable to induce the stringent response) with inhibitors of peptidoglycan synthesis resulted in lysis (Kusser & Ishiguro, 1985). Intriguingly, these studies also found that addition of chloramphenicol promoted lysis in growth-arrested cells, an unintuitive observation given that chloramphenicol itself stops growth and thus increases tolerance. It was proposed that the mechanism of chloramphenicol-dependent lysis was based on the ability of chloramphenicol to "relax" peptidoglycan synthesis in nongrowing mutant *relA* bacteria, i.e. uncoupled lysis from growth arrest. This can likely be attributed to chloramphenicol's inhibitory effect on translation, which might interrupt RelA signaling (which requires an interaction with ribosomes bound to uncharged tRNAs, i.e. active translation, for ppGpp signaling). Another study likewise demonstrated a direct correlation between overexpressed (p)ppGpp and penicillin tolerance in *E.coli* (Rodionov & Ishiguro, 1995). Here, accumulation of ppGpp in the absence of amino acid deprivation was achieved by overexpressing RelA. The overproduction of ppGpp resulted in the inhibition of both peptidoglycan and phospholipid

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synthesis, and consequently in penicillin tolerance, and the addition of chloramphenicol induced lysis (Rodionov & Ishiguro, 1995).

(p)ppGpp inhibits phospholipid synthesis through PlsB (Noga et al., 2020), and these authors found that penicillin tolerance could be reversed by providing more glycerol-3-phosphate, a substrate for PlsB to use to make phospholipids. Thus, these early studies already pointed towards an intriguing connection between membrane properties and PG synthesis. While the mechanistic underpinnings of this connection are still not understood, this connection has recently received renewed interest (García-Heredia, 2023; Willdigg et al., 2023). It is thus possible that the stringent response does not simply cause tolerance by shutting down growth, but that instead there are more nuanced connections between starvation and cell wall turnover. It is possible, for example, that decreased membrane synthesis directly or indirectly inhibits the activity of the primary effectors of β-lactam induced lysis, the autolysins. In addition, the stringent response has been implicated in its ability to mitigate ROS production (a potentially lethal side-effect of β-lactam exposure) via control of catalases (Khakimova et al., 2013; Martins et al., 2018; Nguyen et al., 2011). ROS mitigation could therefore also contribute to tolerance in a growth-rate-independent manner. This would suggest that growth rate might actually be an imperfect predictor of antibiotic susceptibility. Consistent with this idea, cell wall active-antibiotic can actually kill non-growing cells (Eng et al., 1991; Wu et al., 2018), and often fail to kill fast-growing cells (Cross et al., 2019; Dörr et al., 2015). In addition, a recent study proposed that more than growth rate, the "energy state" of the cell determines antibiotic susceptibility (Lopatkin et al., 2019; Stokes et al., 2019). The notion that ATP concentration, and more broadly metabolic state, influences susceptibility to cell wall-active antibiotics will be explored further in the next section.

3. Link between energy state and CWA susceptibility

Adenosine triphosphate (ATP) is the main energy carrier used throughout all forms of life. In bacteria, ATP is generated from glycolysis, fermentation reactions, the TCA cycle, as well as aerobic and anaerobic respiration using a variety of organic and inorganic electron donors and acceptors. The bridge between many of these processes are electron carriers like NAD/NADH, which serve (alongside a role in macromolecule synthesis) as shuttles for electrons extracted from carbon sources like glucose to feed the electron transport chain to maximize ATP generation. The ability to generate ATP and other energy carriers should be limiting for growth and macromolecular synthesis and could thus correlate with CWA susceptibility either indirectly through growth rate modulation (as discussed above), or due to changes in antibiotic target availability or mitigation of antibiotic-induced damage. A recent study uncoupled growth rate and ATP levels within the cell and found that ATP level more accurately predicted antibiotic lethality (including β-lactam antibiotics) than growth rate alone (Lopatkin et al., 2019). ATP as a determinant of antibiotic susceptibility has also been suggested in other studies (Conlon et al., 2016; Y. Wang et al., 2018); the imperfect correlation between growth rate and CWA efficacy has likewise been noted before (Fung et al., 2010). The reason for this observation is mechanistically unclear, but an argument could be made that increased ATP is indicative of increased metabolic activity as a whole. Increased metabolism might indicate increased respiratory activity,

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causing additional damage through ROS upon antibiotic exposure (Dwyer et al., 2009, 2014; Kohanski et al., 2007; Lobritz et al., 2015, 2022; Wong et al., 2022). While the primary mechanism of action of CWAs is still cell wall disruption, increased or decreased metabolic reactions could in principle contribute to either the severity of the disruption (increased autolysin activity might result from an increased rate of cell wall synthesis), or the cell's ability to mitigate indirect sequelae like ROS production. A worthwhile future endeavor will be to determine how the many cells that are highly tolerant to β-lactams despite full susceptibility to the primary mechanism of CWA action survive these perturbations. Many Gram-negative bacteria form fully viable spheroplasts upon β-lactam exposure (Cross et al., 2019; Dörr et al., 2015; Monahan et al., 2014). Such spheroplasts do experience oxidative damage and presumably other metabolic imbalances yet survive at close to 100 % (Shin et al., 2021). In *V. cholerae,* oxidative stress upon β-lactam exposure is transient, indicating perhaps a heightened ability to repair CWA-induced secondary damage. To make matters more complex, however, the same metabolic perturbations (ROS production) that contribute to CWA lethality actually have the opposite effect in some bacteria, i.e. they cause increased resistance. In *P. aeruginosa,* increased NADH levels led to the activation of antibiotic efflux pumps and high levels of antibiotic resistance, however this was a double-edged sword; the increase in NADH also caused an increase in intracellular ROS and thus amplified antibiotic killing (Arce-Rodríguez et al., 2022). In conclusion, the availability of energy intermediates can modulate CWA efficacy, albeit in mechanistically poorly understood ways.

4. Dividing the loot: Metabolic resource allocation and antibiotic susceptibility

Bacterial metabolism consists of several key structural elements that are channeled into various biosynthetic processes. These fundamental building blocks for macromolecule synthesis are either taken up from the environment, or derived from central catabolic processes like glycolysis, TCA cycle or the pentose phosphate pathway, summarized in Fig. 2. For example, peptidoglycan, the Gram-negative O-antigen, enterobacterial common antigen (ECA), and LPS, as well as the Gram-positive functional counterpart teichoic acids, all draw their building blocks from central carbon metabolism. Peptidoglycan biosynthesis branches from glycolysis/gluconeogenesis via fructose-6-phosphate, which is thus part of both central carbon metabolism and a building block for PG precursors. LPS consist of diverse sugars, the lipid A portion stemming from the same glycolytic branch point (F6P), the KDO core from the pentose phosphate pathway (via Ribulose 5-phosphate) integrating with phosphoenolpyruvate from central carbon metabolism, and with O-antigen, consisting of various sugar molecules depending on the bacterial species (Lodowska et al., 2013). ECA consists of colanic acid, whose biosynthesis originates form sugar molecules from fructose/mannose metabolism and central carbon metabolism (again via F6P), which are intrinsically related (Wang et al., 2020). Teichoic acids, similar to LPS, are made from sugar subunits, either GlcNAc or ManNAc, and repeating glycerol or ribitol phosphates, which branch from the pentose phosphate pathway (Swoboda et al., 2010). In addition, several of these processes (PG, O-antigen, ECA, and teichoic acids), also rely on the same molecule, undecaprenol pyrophosphate (Und-PP), for transport across the membrane (Jorgenson & Young, 2016) (discussed further below). Thus, since many essential cell envelope processes

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draw precursors from the same central carbon metabolism pools, variations in such pools (or excessive demand from one of those processes) can alter the effective synthesis of envelope components, resulting in changed CWA susceptibility, and several examples of this exist. Variations in precursor pools can be induced by antibiotics (which send one process into overdrive, see "futile cycling" discussed below), or by growth on a specific carbon source, as observed in *Mycobacterium tuberculosis,* which changes its cell envelope structure during growth on cholesterol (Koh et al., 2022). Here we dissect the junction between precursor channeling and competition, and how balancing precursor availability affects susceptibility to cell wall-active antibiotics.

4.1 Enzymatic inhibition and precursor competition: Glycolysis products

The substantial metabolic flux associated with PG synthesis can be explored when the process is inhibited. The major glycolytic branch point towards PG production is with Fructose-6-phosphate (F6P) (Sachla & Helmann, 2021). F6P is directly shuttled to envelope synthesis by GlmS, then converted into UDP-GlcNAc by GlmMU, and then processed by MurA directing UDP-GlcNAc into PG synthesis. In *E. coli*, UDP-GlcNAc is a shared substrate for MurA and LpxA. LpxA directs UDP-GlcNAc into LPS synthesis with the help of LpxC (Han et al., 2020) as the first committed step towards LPS biogenesis. Thus, glycolytic and gluconeogenic fluxes can in principle have strong modulatory influences on both PG and LPS biosynthesis, which can consequently alter susceptibility to cell wall-active antibiotics. Not surprisingly, the balance between PG precursor flux and LPS biosynthesis is carefully balanced in some bacteria. For example, *Pseudomonas aeruginosa* employs direct interaction between MurA and LpxC, where MurA stimulates LPS biosynthesis to avoid imbalanced production of cell envelope components (Hummels et al., 2023).

The balance between PG synthesis and central carbon metabolism has been particularly well-studied in several model organism. GlmS, the major branchpoint protein between glycolysis and PG precursor synthesis, is under tight regulation. Work in *B. subtilis*, for example, identified a crucial flux regulator (GlmR, aka YvcK) between glucose metabolism and PG synthesis via dual control. First, the product of GlmS, glucosamine-6-phosphate, post-transcriptionally inhibits the *glmS* transcript via riboswitch in the first negative feedback loop (Watson & Fedor, 2011). In the second negative feedback loop, GlmR is inhibited by UDP-GlcNAc accumulation, signaling abundant PG precursors. Since GlmR ordinarily activates GlmS, which controls the metabolic flux between F6P and PG, UDP-GlcNAc accumulation effectively reduces GlmS activity (Patel et al., 2018).

Similar tight feedback control of precursor synthesis flux has been observed in *E. coli*, where GlmS levels are also fine-tuned by GlcN6P sensing in a complex genetic circuit. The small RNA GlmZ positively controls the *glmS* mRNA in response to GlcN-6-P concentrations and GlmZ is negatively regulated by YhbJ (Kalamorz et al., 2007). When GlcN6P is abundant, RNA-binding protein RapZ sequesters GlmZ and targets it for degradation through ribonuclease recruitment. Upon GlcN6P depletion, the decoy sRNA GlmY accumulates and sequesters RapZ, suppressing GlmZ decay and allowing downstream

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expression of GlmS (Khan et al., 2020). These elaborate feedback loops likely ensure that the cell allocates F6P properly between central carbon metabolism and PG synthesis.

Other connections between central carbon metabolism and PG homeostasis exist. Deleting the gene for glucose-6-phosphate isomerase*, pgi*, in *V. cholerae*, activates the cell envelope stress responses system, VxrAB (Keller et al., 2023). Pgi is responsible for converting glucose-6-phosphate (G6P) to F6P and vice versa during glycolysis/gluconeogenesis. Upon *pgi* deletion, the cells accumulate both G6P and F6P and become sensitive to osmotic imbalances and suffer morphological defects. Consistent with cell envelope dysfunction, the mutant also has an increased susceptibility (both decreased resistance and tolerance) towards CWA. Suppressor mutants in the PTS system rescued the mutant, pointing to a role of sugar phosphate stress in these phenotypes. Interestingly, the cell wall deficiency phenotypes could be suppressed by supplementation of extracellular GlcNAc, pointing to putative inhibition of PG precursor synthesis by imbalances in glycolytic/gluconeogenesis flux. It is possible that the accumulation of G6P (which can be easily converted to G1P in the cell) in a *pgi* mutant leads to inhibition of cell wall synthesis, as a previous study demonstrated that GlmM was inhibited by G1P levels in *B. subtilis* (Prasad & Freese, 1974). While further work is needed to solidify the mechanism behind this sugar intolerance, this paper demonstrates the field is only beginning to understand the complexities underlying the connection between bacterial metabolism and antibiotic susceptibility. More broadly, these results suggest that perhaps natural, environment-induced fluctuations in glycolysis and gluconeogenesis could potentially result in differential CWA susceptibility due to sugar phosphate toxicity or other metabolic perturbations associated with defective central carbon metabolism.

GlcNAc is not the only essential component being utilized by several pathways. Amino acids are also necessary for PG synthesis to form the short peptide cross links. A typical PG peptide side stem (in model organisms such as in *E. coli* or *B. subtilis*), consists of L-Ala, D-Glu, meso-diaminopimelate (mDAP), and two D-Ala residues. Variations of this theme are found in other species (including lysine instead of mDAP in most Gram-positive bacteria), but the requirement for unusual (D-enantiomers) amino acids is universal (Turner et al., 2014). DAP in particular becomes the limiting amino acid here, since it is also a precursor for lysine biosynthesis (Garde et al., 2021). Thus, PG synthesis and lysine synthesis compete for the same precursor, which could in principle result in altered PG synthesis when lysine is not available in the environment (and/or when endogenous synthesis fails to keep pace with increased lysine demand). Similarly, competition between aspartate (a precursor for mDAP production) biosynthesis and PG synthesis has been observed in *B. subtilis* (Zhao et al., 2018): Limiting aspartate results in PG synthesis inhibition and concomitant increase in sensitivity to cell wall-active antibiotics. While these experiments were conducted in a mutant background, these observations raise the possibility that physiological fluctuations in the need for aspartate for protein synthesis might likewise modulate PG synthesis and consequently tolerance to cell wall-active antibiotics. In *Caulobacter crescentus*, accumulation of the TCA cycle intermediate alpha ketoglutarate (alphaKG) results in morphological defects and increased CWA susceptibility, likely due to alphaKG competitively inhibiting succinyl diamino pimelic acid aminotransferase, a key enzyme in the DAP synthesis pathway (Irnov et al., 2017). This observation highlights yet another example of how central carbon metabolism converses with cell envelope synthesis.

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In *Salmonella* Typhi, GlmS is regulated by nitrogen levels, more specifically, by glutamine availability (Yoo et al., 2016). In the presence of sufficient nitrogen, dephosphorylated PTS^{Ntr} does not significantly compromise GlmS activity, which, in turn, provides abundant amino sugars for constructing cell walls. When cells suffer from depleted nitrogen, phosphorylated PTS^{Ntr} tightly binds to GlmS and inhibits the enzyme from consuming glucosamine to produce GlcN6P, which slows down LPS and peptidoglycan synthesis. However, GlmS is released from the inhibition by Lon-mediated degradation of PTS^{Ntr} upon depletion of cellular amino sugars (Yoo et al., 2016). Thus, for *Salmonella,* nitrogen availability (in place of, or addition to, central carbon metabolism) appears to be a significant limitation for cell wall synthesis. Collectively, there is thus ample evidence that disrupting central metabolism can affect the processes that are central to cell envelope homeostasis, which suggests that anti-metabolism drugs could be a productive avenue to perturbing the bacterial cell wall and thus increase the efficacy of cell wall-active antibiotics.

4.2 Enzymatic inhibition and precursor competition: The many demands for the undecaprenol carrier molecule

Precursor availability can also be regulated through competition between different biosynthetic pathways. A classic example of this is the battle for undecaprenol phosphate (Und-P) between several cell envelope functions. Und-P has the important role of facilitating the transport (via the specialized translocases MurJ (Meeske et al., 2015) or Amj (Sham et al., 2014)) of the hydrophilic PG precursor lipid II, across the inner membrane, to be incorporated into the growing PG polymer. Crucially, the transport function is facilitated by the pyrophosphate form of undecaprenol (Und-PP), but recycling Und-P for another carrier round requires it to be in its monophosphate form. Loading Und-P with precursors also happens in the cytoplasm, while the Und-PP form is a byproduct of PG synthesis in the periplasm. Thus, bacteria have to both dephosphorylate Und-PP (with the help of phosphatases like BacA and PgpB) (Manat et al., 2014), and translocate the monophosphate back into the cytoplasm (using the newly-discovered translocases PopT, YghB, and UptA (Doerrler et al., 2013; Roney & Rudner, 2023; Sit et al., 2022).

Und-P is highly limiting in the cell, accounting for 270 nmol/g of cell dry weight (Barreteau et al., 2009), yet is shared as a carrier molecule between PG synthesis, O-antigen (Gram-negatives), wall teichoic acids (Gram-positives), capsular polysaccharides, common enterobacterial antigen, and exopolysaccharides (Manat et al., 2014). This creates a potential conundrum for the cell, which needs to ensure that sufficient Und-P quantities are available to drive all envelope biogenesis processes, while balancing this with a presumed trade-off of having surplus Und-P (P) accumulating in the membrane. A runaway function in any one of these pathways can potentially sequester Und-P and make it unavailable to the other processes, and several examples of this happening have been observed. Work from Kevin Young's group, for example, uncovered that a mutation in WecE (an enzyme involved in the synthesis of Enterobacterial common antigen, ECA, an outer membrane component of many Enterobacterales), unexpectedly caused cell enlargement, filamentation and bile sensitivity, suggesting defects in cell wall homeostasis (Jorgenson et al., 2016). Through clever genetic analysis, these authors then discovered that an accumulation of dead-end ECA intermediates resulted in sequestration of Und-P (which could not be properly recycled due to the

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absence of final stages of ECA synthesis). The resulting reduction of Und-P availability subsequently affected PG precursor translocation (Jorgenson et al., 2016). Consistent with this model, the study found that the PG precursor enzyme, MurA, competes with WecA for UDP-GlcNAc, and that overexpressing MurA restored the balance in WecE mutants. This precursor competition also activated multiple stress responses systems such as RCS and Cpx. Since RCS initiates synthesis of colanic acid, its induction also increases demand from Und-P pools. Thus, a balance is needed between different metabolic pathways that require the same starting material, and when this process goes awry, PG synthesis is disturbed, making the bacteria more susceptible to CWA.

Und-P is also required for the translocation of O-antigen precursors. Bacterial Lipopolysaccharide (LPS) is made of three parts: 1) lipid A, a lipidated glucosamine dimer derivative, 2) a relatively short core oligosaccharide, and 3) a distal polysaccharide (O-antigen) (Delcour, 2009). The O-antigen is nonessential in *E.coli* and is an immunogenic molecule that is highly variable amongst Gram-negative bacteria, consisting of 1 to 40 repeating units (Delcour, 2009). The competition for Und-P between O-antigen and PG synthesis has been well-characterized in *E. coli* by Kevin Young's and Matt Jorgenson's group. One study elucidated the effects of disrupting O-antigen biosynthesis and UDP pools within *E. coli* (Jorgenson & Young, 2016). Similar to observations with ECA (discussed further above), mutations in the O-antigen flippase WzxB or the WaaL O-antigen ligase (which fuses O-antigen with LPS core), resulted in swollen cells with division defects. Suppressor mutants that inhibited the synthesis of O-antigen intermediates at the first committed step (connecting Und-P with N-acetylglucosamine) restored viability. Similar to their findings with ECA, it was determined that morphology defects were not the consequence of the mere absence of O-antigen, but rather reflected the accumulation of Und-PP-linked intermediates and thus *de facto* depletion of Und-P (Jorgenson & Young, 2016). Since accumulation of dead-end intermediates of processes requiring Und-P is detrimental to the cell, this could in principle be targeted with antimicrobial therapy to enhance effectiveness of cell wall active drugs. Indeed, Und-P defects are additive with mutations in Penicillin-binding proteins: A enzymatically defective Und-P synthase (mutation in the gene encoding UppS) in combination with mutations in PBPs causes lysis at high temperatures. Lysis can be prevented when *uppS* is restored, or by supplying more PG precursors to the cells through MurA overexpression, or through the overexpression of aPBPs (Jorgenson et al., 2019). The authors also demonstrate the potentiating power of targeting Und-P homeostasis by first inhibiting the cells with fosmidomycin, which inhibits Und-P, followed by treatment with the bPBP2 inhibitor mecillinam. This combination therapy proved more effective than single antibiotic treatment and adds evidence to the benefit of targeting bacterial metabolism. However, Und-P disruptions can have global effects beyond cell wall synthesis. In *B. subtilis* a point mutation in *uppS* spurred vancomycin resistance (Lee & Helmann, 2013). The decreased level of UppS causes the bacterium to become slightly more susceptible to many late-acting cell wall antibiotics, including β-lactams, but significantly more resistant to fosfomycin and D-cycloserine, antibiotics that interfere with the very early steps of cell wall synthesis. The reason for these phenotypes is that impaired *uppS* activates the σ^M regulon, the primary cell envelope

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stress response, which governs resistance against many CWAs (Eiamphungporn & Helmann, 2008; Lee & Helmann, 2013).

UppS activity also affects Gram-positive wall teichoic acids (WTA) homeostasis. Like the Gram-negative outer membrane, WTA play a crucial role in virulence and bacterial division (Swoboda et al., 2010). Synthesis of WTA in *S. aureus* is initiated by TarO and TarA. TarO transfers an N-acetyl-glucosamine-1-phosphate moiety to a Und-P molecule, followed by the transfer of N-acetylmannosamine by TarA (Farha et al., 2015). This glycolipid is fitted with ribitol-phosphate repeats before export to the external surface and attachment to PG. In *Staphylococcus aureus,* a screen for molecules that suppress the antibacterial activity of targocil, an inhibitor of the WTA flippase (TarG), was answered by clomiphene, a widely used fertility drug (Farha et al., 2015). Upon further characterization, the study found that clomiphene inhibited UppS. This antagonism can be interpreted as clomiphene reducing the availability of Und-P, thereby limiting WTA synthesis. At first, the observation that limiting early WTA synthesis mitigates toxicity of inhibition of a later step appears paradoxical. However, inhibition of late steps of WTA synthesis is mostly toxic due to the sequestration of Und-P in dead-end intermediates (Campbell et al., 2012; D'Elia et al., 2009; Sewell & Brown, 2014). Reducing WTA synthesis earlier (through reduction of Und-P) is apparently less detrimental; though speculative, this may suggest that the PG synthesis machinery has higher affinity for Und-P than WTA synthesis, resulting in a bias towards PG synthesis at lower Und-P levels (D'Elia, Millar, et al., 2006; D'Elia, Pereira, et al., 2006). Interestingly, combination therapy of targocil and clomiphene potentiated the activity of β-lactam antibiotics against methicillin-resistant *S. aureus* (MRSA) (inhibition of WTA acid synthesis had also previously been shown to potentiate β-lactams against MRSA (Farha et al., 2013)), once again showcasing the therapeutic potential in understanding the fundamental biology of metabolic precursor allocation.

4.3 "Futile cycling" and the hidden cost of β-lactam exposure

In the 1970s and 1980s, significant research effort was devoted to uncovering the mechanism by which β-lactam antibiotics kill bacteria. Early experiments with a variety of bacteria yielded seemingly paradoxical results. While it seemed clear that autolysins contributed to cell wall degradation and lysis, a *Streptococcus pneumoniae* mutant defective in major autolysin activity still died, but without lysing, in the presence of β-lactams (Horne & Tomasz, 1977). In addition, *Streptococcus* species were identified that naturally died a lysis-free death (Dörr, 2021; Tomasz, 1979). In 2014, nearly 3 decades later, this question was still puzzling the field. New insight then arrived through work in *E. coli.* Here, studies of the Rod system are facilitated by the availability of antibiotic compounds that target specific parts of this cell elongation machinery, e.g. MreB (A22, MP265) and the essential bPBP2 (mecillinam). Interestingly, the Rod system can be rendered non-essential for growth via suppressor mutations, resulting in resistance against MreB inhibitors like A22. Thomas Bernhardt's team discovered that paradoxically, an A22-resistant mutant was still unable to grow in the presence of mecillinam. Thus, it became clear that simple inhibition of the Rod system is distinct from the effects of mecillinam. Even more paradoxically, these cells were resistant against a combination of A22 and mecillinam. Further investigation revealed that rather than just inhibiting PBP2, mecillinam induced a "futile cycle" of

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generation of long, uncrosslinked PG strands, followed by continual degradation by a lytic transglycosylase (Slt70). This process, which was also shown to be induced by diverse other β-lactams, was detrimental. Addition of A22 inhibits Rod system function upstream of PBP2, thereby preventing futile cycling, which confers mecillinam resistance in the suppressor strain. Several other studies are consistent with this model, including a very elegant study on cell wall turnover during cell elongation by Uehara and Park (Uehara & Park, 2008), a follow-up study showing that activation aPBPs prevents mecillinam-induced futile cycling by sequestering PG precursors away from PBP2 (Lai et al., 2017), and several older observations that noted lysis as a consequence of overexpression of PBPs with their TP active site mutated (which should likewise result in futile cycling) (Legaree et al., 2007; Meisel et al., 2003). It is still unclear how futile cycling contributes to β-lactam-induced lethality, but large-scale metabolic alterations have been noted in mecillinam-treated *E. coli* (Lobritz et al., 2022). These alterations might contribute to cell death/growth inhibition, e.g. via increased oxidative stress caused by increased flux into the electron transport chain. It is also possible that the metabolic demand of futile reactions exhausts catalysts required for macromolecular synthesis. However, many bacteria are highly tolerant against β-lactam antibiotics (Cross et al., 2019; Shin et al., 2021), suggesting that they are able to mitigate the toxic effects of futile cycling.

Another indirect effect of futile cycling that affects CWA susceptibility is the fate of PG fragments. Many of the PG fragments generated by futile cycling are either lost to the environment or recycled back into the cytoplasm for re-incorporation into cell wall synthesis (or consumption as a carbon source in some bacteria). Importantly, these fragments also serve as the signal that induces the expression of chromosomal, AmpC-type β-lactamases in many bacteria, thereby inducing resistance against the offending antibiotic (Jacobs et al., 1994, 1997). Mutants in LTGs, which cannot process long PG strands into smaller fragments, cannot generate this signal and are therefore often more susceptible to β-lactams (Cho et al., 2014; Figueroa-Cuilan et al., 2022; Korsak et al., 2005; Kraft et al., 1999; Weaver et al., 2022). However, even in bacteria that do not encode β-lactamases, e.g. *V. cholerae* and non-AmpC-producing *E. coli*, LTG mutants exhibit enhanced killing by β-lactams. In *V. cholerae,* this was shown to be the consequence of uncrosslinked PG strands accumulating in the periplasm, which kills cells in a poorly-understood way (Weaver et al., 2022). Thus, both futile cycling and the inability to engage in it are detrimental, showcasing once more the incredible versatility of β-lactams as some of our most powerful antibiotics. For a more in-depth discussion of the resource distribution between central metabolism and cell envelope synthesis, we refer the reader to a recent excellent review on the topic (Sachla & Helmann, 2021).

5. Metabolism in the infection environment

Bacteria are sure to behave differently on a lab bench compared to an infection environment. Pathogens often rely on their hosts for many essential nutrients and the host exploits this to fight back, while the bench scientist strives to keep their living tools happy and full of nutrients. We explore the metabolic complications that can arise during an infection, and how this may affect susceptibility to CWAs. Few studies are available that directly address

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CWA susceptibility during an infection, but many of the pathways outlined above are highly likely to also modulate antibiotic efficacy during infection.

5.1 Nutrient Deprivation: amino acids

The human innate immune response has an arsenal of tools at its disposal that make it harder for bacteria to gain a foothold. One such tool is amino acid deprivation. While the host tries to slow down bacterial growth by restricting the availability of nutrients, bacteria mount a counterattack and try to maximize their access to them. Amino acids like arginine, histidine, asparagine, and tryptophan, as well as branched chain amino acids (BCAA) like Ile, Leu, and Val are central points of competition between the host and pathogen (Kaiser & Heinrichs, 2018; Ren et al., 2018). It's been shown that concentrations of these amino acids in specific niches during infection is critical to sustain growth (Zhang & Rubin, 2013). As a way to potentiate antibiotic effectiveness, one could shut down the expression of these amino acid biosynthetic pathways. Taking advantage of the restriction of nutrients by the host immune response and targeting nutrient biosynthesis is a promising avenue for antibacterial development. In particular, amino acids like lysine and glutamine, which are essential in the synthesis of PG, are of high priority for new targets. However, as described above, amino acid starvation also induces the stringent response, potentially causing unintended induction of CWA tolerance.

5.2 Bacterial Metal Homeostasis and CWA efficacy

Another weapon wielded by the innate immune response is metal sequestration and intoxication (sometimes alternating between the two approaches), in particular, manganese, iron, and zinc (Merchant & Helmann, 2012). Metal homeostasis is an important regulatory process in nearly all of life, but especially in bacteria, where metals become toxic in high quantities but are necessary for numerous essential reactions to occur. In bacteria, it is estimated that 30 to 45% of enzymes require a metal co-factor for function (Klein & Lewinson, 2011). Respiration, DNA replication, photosynthesis, and nitrogen fixation all are dependent on metal availability (Merchant & Helmann, 2012). Metals cannot be synthesized or degraded within the cell, and thus the balance relies on the modulation of transport into and out of the cell (Chandrangsu et al., 2017). Consequently, metalloregulatory systems exist within bacteria to respond to metal intoxication and metal starvation. Since metal homeostasis is also connected to cell wall homeostasis (many autolysins, for example, are metallo-enzymes), metal availability can alter susceptibility to CWAs (Lonergan et al., 2019). As a side-note, some metals (e.g., zinc) can directly inactivate certain antibiotics, such as penicillin and tetracyclines (Doluisio & Martin, 1963; Eisner & Porzecanski, 1946), outside of the role of metal homeostasis in bacterial physiology. In this section, we will outline how metal homeostasis affects processes that can potentially modulate CWA efficacy in the host.

5.3 Metal Starvation and CWA susceptibility

Immediately after invasion by infecting bacteria, the body will initiate a nutritional immune response, sequestering trace metals. This response typically aims at slowing or stopping microbial growth. For example the host protein, calprotectin, sequesters zinc and manganese at sites of infection (Corbin et al., 2008). Host-produced transferrin and lactoferrin sequester

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iron (Murdoch & Skaar, 2022), thereby limiting the pool of free iron available for infecting bacteria to take up. In addition, the host protein lipocalin binds to the siderophores (small molecules with high iron affinity that are imported via transporters in the bacterial cell envelope) that bacteria use to sequester iron from their environment (Baichoo et al., 2002; Flo et al., 2004). In return, bacteria induce numerous responses with the goal of maximizing metal acquisition in limiting environments. This is often accomplished through specialized uptake systems. To counteract iron starvation, for example, bacteria produce siderophores, other iron uptake systems (e.g., ABC transporters for the import of ferrous or ferric iron), and more indirect pathways like cytotoxin production for the destructive release of iron from blood cells (Chandrangsu et al., 2017). Zinc starvation is counteracted by both high affinity and low affinity zinc uptake systems. Since metal intoxication is as much of a risk as metal starvation, these systems are usually tightly controlled through starvation sensors. The canonical bacterial response to starvation is induction of the Fur regulon, which is highly responsive to decreases in intracellular iron levels, and controls siderophores and other iron uptake systems (Baichoo et al., 2002). Zinc starvation is sensed by Zur, which controls zinc uptake, but also zinc-independent replacement enzymes for ordinarily zinc-dependent processes to reduce intracellular sequestration of this trace metal (Ducret et al., 2022; Lonergan & Skaar, 2019; Palmer & Skaar, 2016).

Metal starvation can impact CWA efficacy in multiple ways. In an unusual example, the antibiotic itself can actually induce a metal starvation phenotype, as demonstrated in vancomycin sequestering zinc (Zarkan et al., 2016). This could in principle contribute to vancomycin's efficacy, and this contribution would vary with zinc availability in the infection environment. The availability of metals can also affect proteins required for mediating resistance against CWAs. Metallo-β-lactamases (e.g., NDM-1) require zinc to function, and their ability to confer full resistance is thus sensitive to zinc starvation (Bebrone, 2007; Carfi et al., 1995; Daiyasu et al., 2001; McCall et al., 2000; Padyab et al., 2020). Since zinc starvation responses seek to improve intracellular zinc levels, and NDM-1 is extracellular (periplasmic), the carbapenemases might actually disproportionately be affected by low zinc levels.

The most prominent effect, however, is probably due to metal starvation inducing growth arrest. Growth arrest, as we have outlined above, can trigger CWA tolerance, a case of the immune system inadvertently antagonizing antibiotic efficacy. The degree to which this is relevant for the bacterium likely depends on its ability to prevent metal starvation, i.e. it is a multifactorial function of a bacterium's ability to induce uptake systems vs. the host's ability to robustly sequester metals.

Metal starvation is also more directly linked with CWA efficacy. Cell wall autolysin activity can be induced by metal starvation (Lonergan & Skaar, 2019; Murphy et al., 2019), potentially altering efficacy of CWAs that rely on autolysins for their lethal action, e.g. the β-lactams. In *V. cholerae*, the PG endopeptidase ShyB is under control of the zinc starvation regulon (Murphy et al., 2019). Under high zinc concentrations, the expression of ShyB is repressed; conversely, the enzyme is induced during zinc starvation. This raises the possibility that ShyB might potentiate β-lactam antibiotics, i.e., that those agents would be more effective under zinc starvation conditions. Crucially, this type of zinc regulation

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is observed in other diverse Gram-negative bacteria, including the plague-causing *Yersina pestis,* and the nosocomial pathogen *Acinetobacter baumannii* (Lonergan et al., 2019; Pradel et al., 2014). In the latter, zinc starvation synergizes with CWA efficacy; however, this was not dependent on the zinc-dependent endopeptidase, and differed between different classes of antibiotics (Lonergan et al., 2019). Thus, more work is needed to determine the exact mechanistic connection between zinc starvation and CWA efficacy. Lastly, metal starvation can have broad impacts on central metabolism, with potentially associated altered CWA susceptibility: *M. tuberculosis,* for example, exhibits perturbed cholesterol catabolism upon iron starvation (Theriault et al., 2022). As discussed further above, cholesterol catabolism also interfaces with cell envelope structure, potentially altering *M. tuberculosis's* response to antibiotics.

5.4 Metal Intoxication and CWA susceptibility

Metal intoxication as an antimicrobial strategy is extensively used by phagocytes. Following phagocytosis, the phagolysosome, for example, is pumped full of copper ions to aid in bacterial killing. A key part of the innate immune response is generation of reactive oxygen species (ROS) and reactive nitrogen species (NOS), which are potentiated by the presence of metals, in particular iron and zinc (through its effect on NADPH oxidase) (Beard et al., 1995; Braymer & Giedroc, 2014). ROS and metals also interact within bacterial cells in other ways. In *E.coli,* for example, upon ROS production, iron and zinc ions accumulate intracellularly, and these extra ions can mismetallate metalloproteins, such as the copper sensing CsoR or the heme regulator Per, with an incorrect metal not suited for the enzyme to function properly, leading to heme toxicity, or increased ROS (Anjem et al., 2009; Anjem & Imlay, 2012; Chandrangsu et al., 2017; Chandrangsu & Helmann, 2016; Gaballa et al., 2012; Imlay, 2014; Ma et al., 2009; Varghese et al., 2007). The resulting growth arrest (at least *in vitro*) may again affect CWA efficacy. Excess metals can also disrupt central carbon metabolism. Zinc toxicity in *Streptococcus pyogenes,* for example, results in inhibition of glycolytic enzymes and phosphoglucomutase, impairing capsule synthesis (Ong et al., 2015). To overcome this metabolic inhibition, the bacteria switch preferred carbon sources from glucose to galactose, bypassing the zinc-disrupted glycolytic enzymes. As outlined above, central carbon metabolism and specifically glycolysis are linked with cell envelope homeostasis in some organisms (Keller et al., 2023; Tuckman et al., 1997; Zhou et al., 2022), suggesting an indirect way of metal surplus affecting CWA efficacy.

As outlined above for metal starvation, bacteria have also evolved elaborate regulatory systems to prevent metal intoxication. This is particularly well-understood through work by the Helmann group, using *B. subtilis* as a model system. Efflux pumps are a primary means of removing excess and potentially toxic iron, manganese and zinc (Chandrangsu et al., 2017, 2019; Sachla & Helmann, 2019). *B. subtilis* can also buffer excess metals by having a liable pool, with impressive storage capabilities. Upon increased iron exposure, bacteria store this Fe(III) in ferritin (Andrews, 1998), temporarily stabilizing iron levels to provide sufficient time to express more sustainable countermeasures (like efflux pumps) and adapt to the metal stress. Excess zinc can be buffered by bacillithiol (BSH) (Lee et al., 2007; Newton et al., 2009), which also plays a role in detoxifying ROS. Since ROS are a consequence of CWA exposure (Kohanski et al., 2007; Shin et al., 2021), and zinc concentrations might

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contribute to ROS toxicity, BSH is a potentially central molecule for CWA efficacy, though this remains to be explored.

5.5 Macrophages vs Intracellular Pathogens

Phagocytes (macrophages, neutrophils and dendritic cells) are a crucial part of the innate immune response. Phagocytes clear pathogens by engulfment, followed by digestion in a compartment called the phagolysosome. The phagolysosome is an intentionally hostile environmental for bacteria, as the pathogen is bombarded with ROS, low pH, proteases, antimicrobial peptides, metal intoxication, metal starvation, and nutrient limitation (Uribe-Querol & Rosales, 2017). As we discussed above, many of these stresses can cause growth arrest, and thus inadvertently tolerance to cell wall active antibiotics. Indeed, several bacteria exhibit increased tolerance upon phagocytosis, including *Salmonella typhi* and *S. aureus* (Bearson et al., 1998; Hommes & Surewaard, 2022; Ibarra & Steele-Mortimer, 2009; Peyrusson et al., 2020). Within 30 min after phagocytosis by macrophages, *Salmonella* cells follow one of two fates, either replication and generation of virulence factors, or to remaining viable but become nonreplicating persisters, which are highly antibiotic tolerant. However, even the persisters are still able to produce virulence factors (Stapels et al., 2018), making this particularly nefarious. The stressful environment of the macrophage was found to be the driver behind *Salmonella* persister formation through activation of endogenous toxin-antitoxin modules (Helaine et al., 2014).

In *S. aureus,* ROS, generated by macrophages via respiratory burst, attack bacterial iron-sulfur (Fe-S) cluster containing proteins, including TCA cycle enzymes, resulting in decreased respiration, lower ATP and consequently increased antibiotic tolerance, both in isolated macrophages, and in a mouse model (Rowe et al., 2020). Intracellular *S. aureus* can also exhibit a form of induced tolerance through antibiotic exposure (Peyrusson et al., 2020). In this study, the persister cells remain metabolically active but display an altered transcriptomic profile consistent with activation of stress responses, such as the stringent response, cell wall stress, SOS and heat shock. These changes occur after exposure to oxacillin, clarithromycin, or moxifloxacin.

The world champion of drug tolerance is probably *M. tuberculosis,* likely due to its unusual metabolism coinciding with very slow *in vitro* and *in vivo* growth rates and reduced susceptibility to numerous antibiotics (Goossens et al., 2020; Samuels et al., 2022). In addition to the intrinsic protection by slow growth, *M. tuberculosis* also typically responds to drug pressure by further reduced growth rates, metabolic shifting, and the promotion of efflux pump activity (Goossens et al., 2020). After exposure to antibiotics, *M. tuberculosis's* lipid metabolism and redox homeostasis undergo a metabolic shift, with reduced tricarboxylic acid cycle activity in favor of lipid anabolism (Goossens et al., 2020). Increased lipid anabolism plays a role in cell wall thickening, which slows the uptake of lipophilic drug agents and reduces the entry of hydrophilic drugs, including CWAs, through mycobacterial porins (Goossens et al., 2020).

Taken together, these studies demonstrate that the infection environment can modulate antibiotic tolerance and drug-drug interactions. Models of antibiotic efficacy should thus

also always consider the specific environment pathogens find themselves in when infecting a human host.

6. Conclusions

In summary, bacterial metabolism modulates antibiotic susceptibility in complex ways. Metabolic state contributes to the bacterial cell's ability to sense antibiotic stress, and to properly allocate resources towards repairing damage. Especially for more subtle manifestations of differential antibiotic susceptibility (particularly tolerance), bacterial metabolism constitutes an attractive target for future antibiotic adjuvants. However, the extent to which this contributes to treatment outcomes has only begun to be understood, as most work has been done in model bacteria grown under well-controlled laboratory conditions. Future work should be directed towards gaining a deeper understanding of the diversity of bacterial metabolism in infection contexts (Russell, 2019).

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Abbreviations:

PG	peptidoglycan
CWA	cell-wall acting antibiotic
GT	glycotransferase
TP	transpeptidase
PBP	penicillin binding protein
WTA	wall teichoic acids
ROS	reactive oxygen species
NOS	reactive nitrogen species
BCAA	branched chain amino acids
ATP	adenosine triphosphate
AMP	adenosine monophosphate
NAD	nicotinamide adenine dinucleotide
NADH	1,4-dihydronicotinamide adenine dinucleotide
SOS	sudden DNA damage response system
ppGpp	guanosine 5'-diphosphate 3'-diphosphate
pppGpp	guanosine 5'-triphosphate 3'-diphosphate

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GDP	guanosine diphosphate
GTP	guanosine triphosphate
PRPP	phosphoribosyl pyrophosphate
IMP	inosine monophosphate
MDR	multi-drug resistant
TCA	tricarboxylic acid
LPS	lipopolysaccharide
ECA	enterobacterial common antigen
KDO	3-deoxy-D-manno-octulosonic acid
F6P	fructose-6-phosphate
G6P	glucose-6-phosphate
G1P	glucose-1-phosphate
PTS	phosphotransferase system
DAP	diaminopimelic acid
Und-P	undecaprenyl phosphate
Und-PP	undecaprenyl pyrophosphate
UDP	uridine diphosphate
GlcN6P	glucosamine-6-phosphate
GlcNAc	N-acetylglucosamine
MurNAc	N-acetylmuramic acid
LTG	lytic transglycosylase

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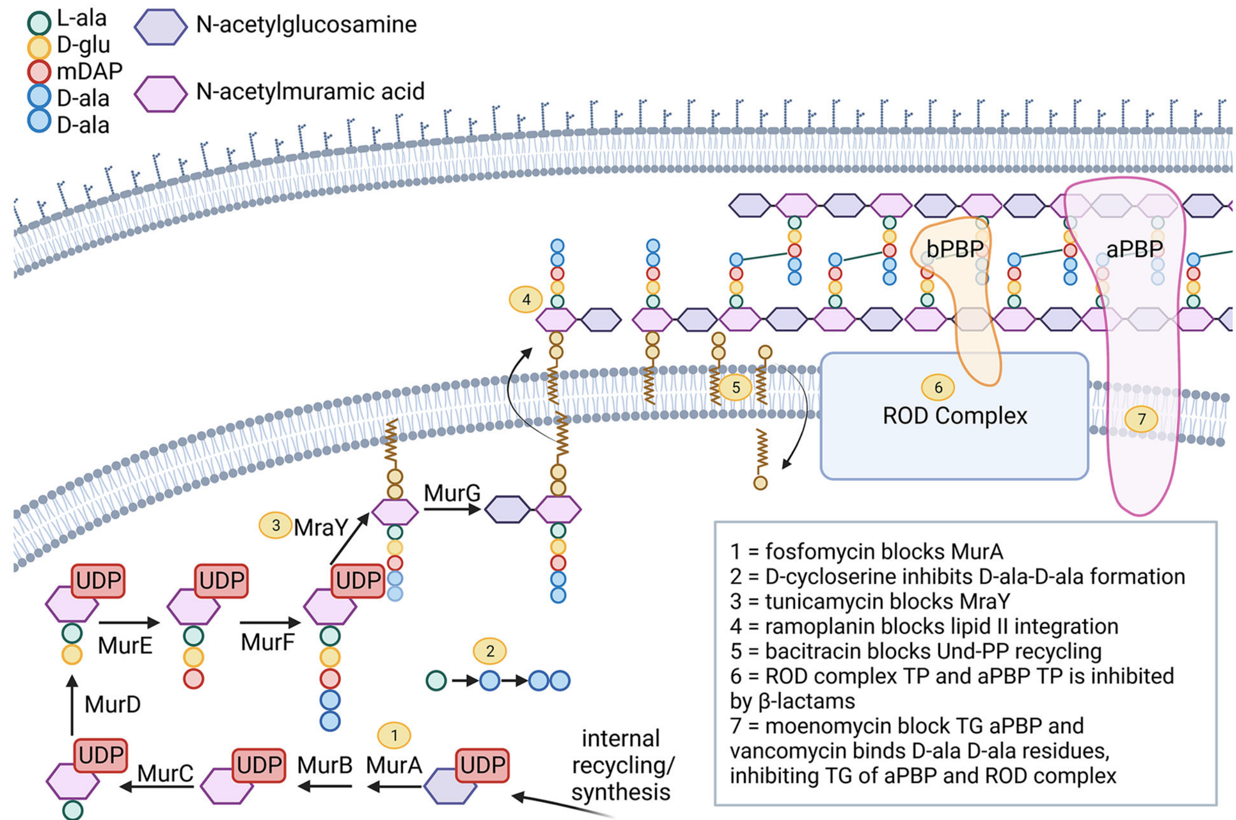

Figure 1:

Schematic depicting common CWA targets during peptidoglycan synthesis in the gram-negative E. coli. UDP-N-acetylglucosamine (UDP-GlcNAc) is converted to UDP-N-acetylmuramic acid (UDP-MurNAc) through the enzymatic activity of MurA and MurB. Fosfomycin inhibits MurA activity by binding the cysteine residue in the active site. MurC attaches the first peptide side stem, L-alanine to UDP-MurNAc. MurD attaches a D-glucose residue to the growing chain. MurE attaches mDAP (L-lysine) to the D-glucose residue. MurF adds the final two, D-alanine residues. D-cycloserine inhibits the conversion of L-alanine to D-ala-D-ala by acting as a cyclic analogue of D-alanine. MraY attaches the UDP-MurNAc + peptides to the lipid carrier resting in the inner membrane. Tunicamycin blocks the activity of MraY, specifically blocking N-linked glycosylation. MurG links GlcNAc to MurNAc+peptides. The lipid II is flipped into the cytosolic space to be integrated into the mature PG. Ramoplanin inhibits the TG activity on lipid II. Bacitracin inhibits Und-PP recycling by preventing the final dephosphorylation step in the phospholipid carrier cycle. The ROD complex, composing of RodA, MreB, MreC, RodZ, and PBP2, carries of TP and TG activity on the growing PG. TP is inhibited by β-lactams, mimicking the D-ala residue, and blocking the active site of PBPs. Moenomycin specifically blocks TG of aPBPs.

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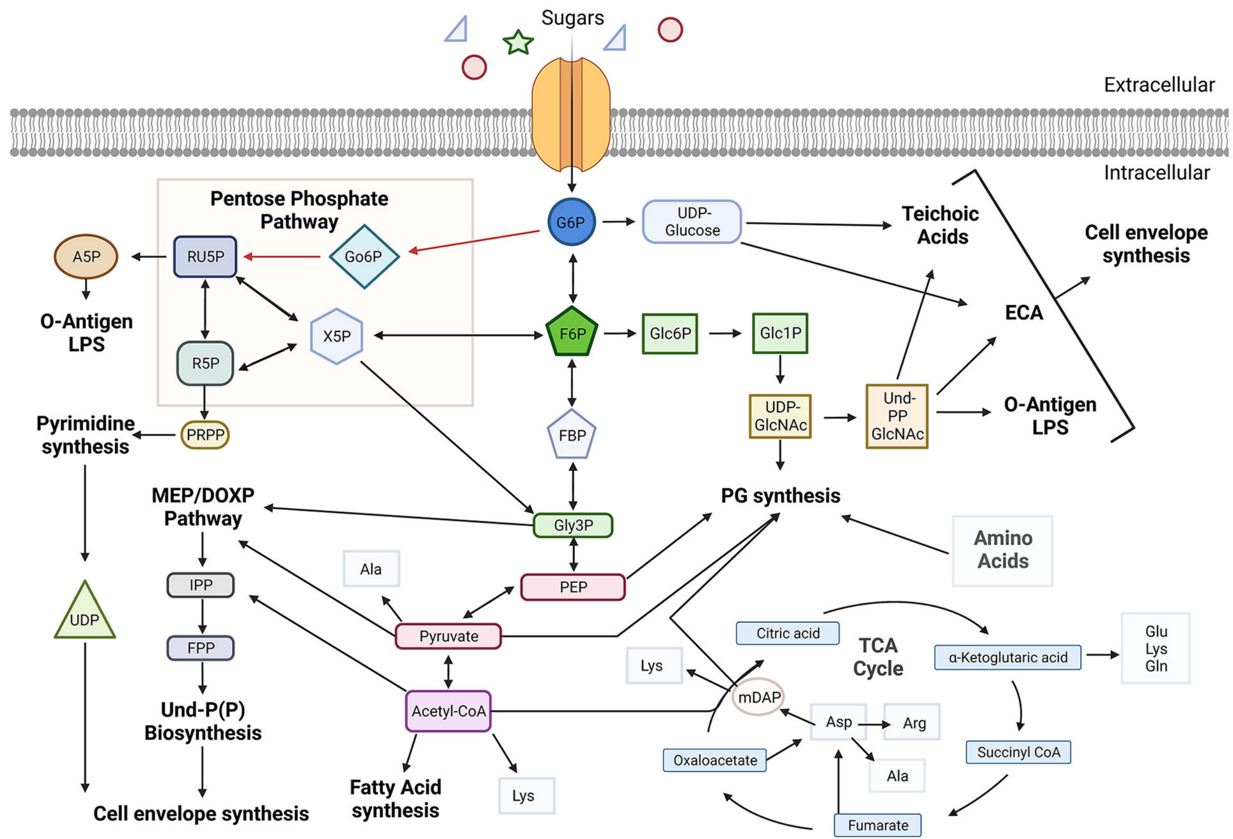

Figure 2:

Metabolic resource connections between central metabolism and cell envelope synthesis. For simplicity's sake, we only consider glucose import as the central carbon source. Sugar import initiates the glycolytic process, converting glucose into glucose-6P (G6P), then fructose-6P (F6P), then fructose-1-6-bisphosphate (FBP), then glyceraldehyde-3P (Gly3P) and phosphoenol pyruvate (PEP). There, it is further processed into pyruvate and acetyl-CoA for integration into the Tricarboxylic acid cycle (TCA). Amino acids key to PG synthesis are shown with Lys (lysine), Ala (alanine), Asp (aspartate), Arg (arginine), Glu (glutamate/glutamic acid), and Gln (glutamine). These amino acids, plus mDAP, can be incorporated into the side stem of PG in many species. The pentose phosphate pathway generates intermediates gluconate-6P (Go6P), ribulose-5P (RU5P), ribose-5P (R5P), xylulose-5P (X5P) and 5-Phospho-alpha-D-ribose 1-diphosphate (PRPP). Arabinose-5P (A5P) is made from RU5P and is incorporated into O-antigen/LPS. PRPP generates nucleotides and uridine-diphosphate (UDP) is highly important in cell envelope synthesis. G6P feeds into PPP in the oxidative route (labeled in red), while F6P enters through the nonoxidative path. F6P is the main shuttle towards PG precursor synthesis through glucosamine-6P (Glc6P) and glucosamine-1P (Glc1P) towards UDP-GlcNAC and Und-PP GlcNAc. These are shuttled towards numerous cell envelope synthesis pathways. In most bacteria, Und-PP is generated through the MEP/DOXP pathway of terpenoid biosynthesis, stemming from Gly3P and Acetyl-CoA. Isopentenyl-PP (IPP) and Farnesyl-PP join to create Und-PP.