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Resource sharing between central metabolism and cell envelope synthesis

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Abstract

Synthesis of the bacterial cell envelope requires a regulated partitioning of resources from central metabolism. Here, we consider the key metabolic junctions that provide the precursors needed to assemble the cell envelope. Peptidoglycan synthesis requires redirection of a glycolytic intermediate, fructose-6-phosphate, into aminosugar biosynthesis by the highly regulated branchpoint enzyme GlmS. MurA directs the downstream product, UDP-GlcNAc, specifically into peptidoglycan synthesis. Other shared resources required for cell envelope synthesis include the isoprenoid carrier lipid undecaprenyl phosphate and amino acids required for peptidoglycan cross-bridges. Assembly of the envelope requires a sharing of limited resources between competing cellular pathways and may additionally benefit from scavenging of metabolites released from neighboring cells or the formation of symbiotic relationships with a host.

Graphical abstract

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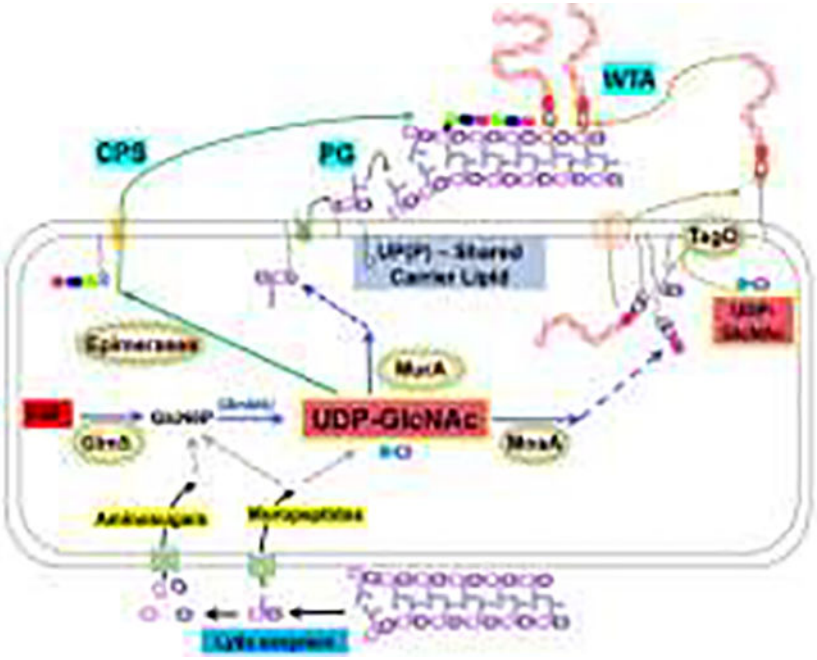

Keywords

UDP-GlcNAc; peptidoglycan; teichoic acid; lipid II; capsule; GlmS; MurA; branch point regulation; PASTA kinase; UPD-GlcNAc

Introduction

"There is no delight in owning anything unshared." ~Seneca (1st century A.D.)

The metabolic pathways that sustain life are a deeply entangled network of reactions that allow the efficient conversion of nutrients into energy and biomass [1]. Detailed reconstructions of metabolism in *Escherichia coli* include more than 2700 reactions that link ~1200 metabolites, with a small subset serving as key nodes for metabolism or as global regulatory signals [2]. Building a cell envelope is a resource-intensive process and imposes a substantial metabolic burden. Gram-positive peptidoglycan (PG) is multi-layered and can represent >20% of cell dry weight [3]. Although the PG layer in Gram-negative bacteria is thinner, the cell envelope additionally includes an outer membrane. Many Gram-positive bacteria also elaborate wall-linked capsular polysaccharide (CPS) and extracellular polysaccharides (EPS). The substantial metabolic flux associated with PG synthesis is unmasked when this process is blocked by antibiotics. Cell wall deficient L-forms of *B. subtilis* (which emerge upon interruption of PG synthesis) have increased carbon flux into lower glycolysis and the TCA cycle, which enhances flux through the electron transport chain and triggers oxidative stress [4].

Here, we highlight the regulation of the key branchpoint enzymes GlmS and MurA. GlmS controls the entry of fructose-6-phosphate (F6P) into envelope synthesis, and MurA directs UDP-GlcNAc into PG synthesis. PG also relies on other shared metabolites (glutamine,

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acetyl-CoA, UTP) and a shared anchor lipid, undecaprenyl phosphate (UP). Cells can also acquire metabolites for cell wall synthesis from their neighbors, and in extreme cases symbiotic partners may share the burden of synthesizing the enzymes required for PG synthesis.

Balancing glycolysis with synthesis of aminosugars.

PG is a glycopolymer comprised of two aminosugars, N-acetyl-glucosamine (GlcNAc) and N-acetyl-muramic acid (MurNAc). Aminosugar biosynthesis initiates when the transaminase GlmS converts F6P to glucosamine 6-phosphate (GlcN6P) using glutamine as the amino donor. Subsequent steps catalyzed by GlmM (mutase) and GlmU (bifunctional acetyl/uridine transferase) generate the key branchpoint intermediate, UDP-GlcNAc (Fig. 1A).

As befits its role as an essential branchpoint enzyme, GlmS is highly regulated. In *Salmonella* Typhimurium, GlmS activity is regulated by nitrogen status, consistent with the requirement of glutamine (Gln) as amino donor. Regulation involves a nitrogen-metabolic phosphotransferase system, PTS^{Ntr}, analogous to PTS systems involved in sugar import. The PTS^{Ntr} EI enzyme is phosphorylated under low nitrogen conditions (signaled by a high α-ketoglutarate/Gln ratio), and this phosphoryl group is transferred through NPr (an HPr paralog) to $EIIA^{Ntr}$. The resulting EII^{Ntr}~P sequesters GlmS in an inactive complex, thereby shutting down aminosugar synthesis (Fig. 1A). When Gln availability is high, EIIA(Ntr) is dephosphorylated and degraded by Lon protease, thereby relieving GlmS inhibition [5].

GlmS production may also be feedback regulated by its product, GlcN6P (Fig 1B,1C). In *E. coli*, the mRNA of the bicistronic *glmUS* operon is processed to yield an unstable *glmS* mRNA, which in turn is stabilized and translationally activated by the action of a small regulatory RNA (sRNA), GlmZ [6] (Fig. 1C). GlmZ is unstable, being targeted for degradation by RNase E by the adaptor protein RapZ [7]. The induction of GlmS expression relies on a decoy sRNA, GlmY, which also binds RapZ. In addition to its role as an RNase E adaptor, RapZ serves as the sensor GlcN6P [8] (Fig. 1C). When GlmS activity is low, GlcN6P-free RapZ indirectly activates transcription of the *glmY* decoy sRNA. The net result is that GlmS translation responds sensitively to changes in the GlmS product, GlcN6P.

GlmS regulation in *Bacillus subtilis* is also complex. The first level of feedback regulation occurs when the *glmS* ribozyme cleaves and inactivates the *glmS* mRNA in response to the GlmS product, GlcN6P [9] (Fig. 1B). Additional control is provided by the GlmR(YvcK) regulator. GlmR stimulates GlmS activity when not bound to the downstream metabolite UDP-GlcNAc [10,11]. GlmR is required for growth on gluconeogenic carbon sources [12], where low F6P levels limit GlmS activity [13] (Fig. 1A). This requirement for GlmR can be bypassed by mutations that inactivate the *glmS* ribozyme or by exogenous aminosugars [14]. When bound to UDP-GlcNAc, GlmR associates instead with a RapZ homolog, YvcJ (Fig 1A) [10,11]. GlmS may also be regulated by ClpCP-dependent proteolysis [15]. Collectively, these mechanisms ensure that GlmS partitions sufficient carbon from glycolysis/gluconeogenesis into aminosugar biosynthesis for cell wall synthesis. A *glmR/yvcK* homolog is essential in *Staphylococcus aureus* [16], and mutants in *Listeria monocytogenes* (*yvcK*) and *Mycobacterium tuberculosis* (*cuvA*) are defective in cell wall

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biosynthesis [17,18]. However, it is unclear if all GlmR family members have this same mechanism.

UDP-GlcNAc is a precious cargo.

The next key branchpoint intermediate is UDP-GlcNAc, a shared metabolite used by MurA in the first committed step of PG biosynthesis (Fig. 2). In Gram-positive bacteria, UDP-GlcNAc is also used for synthesis of the cell surface glycopolymers wall teichoic acid (WTA) and CPS [19,20]. WTA is a long copolymer consisting of glycerol-P (or ribitol-P) units and is covalently linked to PG by a short linker often comprised of GlcNAc and N-acetylmannosamine (ManNAc) [21–23]. UDP-GlcNAc can also serve as a donor for WTA glycosylation [24,25]. UDP-GlcNAc is also the sugar donor for the synthesis of bacilllithiol (BSH), comprised of cysteine, D-glucosamine, and malic acid. BSH is the major low molecular weight thiol in many Gram-positive bacteria and can be present at millimolar levels [26].

The mechanisms that balance the flux of UDP-GlcNAc between PG synthesis and competing pathways have been studied in detail in *L. monocytogenes*. The conserved, multimeric GpsB protein functions as a cell cycle regulator by interacting with penicillin-binding proteins (PBPs) and scaffolding the assembly of multi-protein complexes [27–29] (Fig. 2). Genetic analysis revealed that *gpsB* mutations are suppressed by increasing the level of MurA through loss of the ClpCP protease, or decreasing activity of other enzymes that compete for UDP-GlcNAc [30]. These competing enzymes include GtcA and LMO2550 (involved in GlcNAc decoration of WTA) and MnaA (generates UDP-N-acetylmannosamine in support of WTA synthesis). In *B. subtilis,* the major MurA isozyme (MurAA) is also degraded by ClpCP, but whether this serves a regulatory role is not yet resolved [31].

Capsular polysaccharide (CPS) helps pathogens evade complement fixation, opsonization, and phagocytosis [32]. CPS production initiates with UDP-GlcNAc, a parent compound for capsule building blocks such as UDP-NAc-fucosamine and UDP-D-N-mannosaminuronic acid (Fig. 2). CPS is often subject to nutritional regulation. For example, *S. pneumoniae* grown in galactose (abundant in respiratory mucus) or GlcNAc have the highest level of capsule, followed by glucose or sucrose. The lowest levels of CPS were observed during growth on fructose due to decreased pools of the UDP-sugar precursor [33]. Mutations that affect metabolism often have pleiotropic effects. In the case of *S. pneumoniae* lysine decarboxylase (*cadA*), deletion indirectly affected glycolysis and in turn caused reduced UDP-sugars and impaired CPS biosynthesis [32].

In *E. coli*, UDP-GlcNAc is a shared substrate for MurA and LpxA. LpxA directs UDP-GlcNAc into LPS synthesis by conjugation to an R-3-hydroxyacyl chain. However, the next reaction (LpxC deacetylase) is the committed step for the LPS pathway. LpxC activity is reduced by FtsH-dependent proteolysis when LPS is in excess [34–36]. Proteolysis is also central to the regulation of LPS synthesis in *Francisella tularensis.* In this microbe, RipA is a conserved membrane protein that stabilizes LpxA to direct entry of GlcNAc into LPS biosynthesis [37].

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Catch and release: PASTA kinases coordinate envelope biogenesis.

Many Gram-positive bacteria contain eukaryotic-like serine/threonine kinases (eSTK) with extracellular PBP and serine/threonine kinase associated (PASTA) domains [38]. These signaling kinases may respond to peptidoglycan-associated muropeptides [39,40] and, in at least some systems, directly regulate PG synthesis. In *Listeria monocytogenes,* the PrkA kinase regulates a protease adaptor protein, ReoM, that activates ClpCP-dependent degradation of MurA, thereby reducing PG synthesis. Activation of PrkA by muropeptides leads to ReoM phosphorylation, thereby decreased MurA degradation [41]. PASTA kinases are also critical for cell envelope homeostasis in *M. tuberculosis* [42], where PknB phosphorylates proteins related to PG synthesis including GlmU [43] and CwlM, an essential intracellular cell wall amidase homolog. CwlM localizes to the membrane and regulates the lipid II flippase MurJ. However, upon phosphorylation CwlM~P localizes to the cytoplasm where it activates MurA [44,45] (Fig. 3). PknB also regulates outer membrane mycolic acid synthesis [46,47].

Walls and bridges: diversion of amino acids to PG.

In addition to aminosugars, PG synthesis requires amino acids for the peptide crosslinks. A typical pentapeptide, such as in *E. coli* or *B. subtilis*, consists of L-Ala, D-Glu, meso-diaminopimelate (mDAP), and two D-Ala residues. However, there is some variation in other organisms [48]. Both Ala and Glu are abundant amino acids, and racemases facilitate the interconversion of the L- and D-isomers. Meso-diaminopimelate (mDAP) is a precursor on the pathway to lysine.

Mutations that perturb pentapeptide synthesis can trigger morphological abnormalities or cell lysis. In *Caulobacter crescentus* mutants lacking the RNA chaperone Hfq are slow growing and morphologically altered [49]. Genetic studies traced this defect to decreased activity of TCA cycle enzymes. The resulting increase in α-ketoglutarate (KG) inhibited succinyldiaminopimelate aminotransferase (KG is a product of the reaction), an enzyme required for mDAP biosynthesis [49]. A similar stalling of PG synthesis was observed in a *B. subtilis* strain lacking aspartate transaminase (AspB), the first enzyme in Asp biosynthesis, when grown in rich medium. Asp is a precursor for mDAP biosynthesis, and limitation therefore compromises PG synthesis [50].

Crossing the border: undecaprenol phosphate as a carrier lipid.

Undecaprenyl phosphate (UP) is a C_{55} isoprenoid lipid needed to shuttle hydrophilic envelope precursors across the membrane [51] (Fig. 2). UP serves as a lipid anchor during assembly of PG, LPS O-antigen, and WTA [20]. UP is synthesized in its pyrophosphate form (UPP), and PG transglycosylases also release UPP. Therefore, phosphatases are required for UP synthesis and recycling. The total number of UP(P) carrier molecules in the cell is limited, with an estimated ~1.5×10^5 copies per cell in *E. coli* and *S. aureus* [52]. The UP lipid carrier is coupled to PG precursors to generate the lipid II donor for PG synthesis, and then released and dephosphorylated after transglycosylation. This cycle is estimated to take ~90 seconds, with the flipping of UP(P) back to the cytosol likely rate-limiting [53].

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Depletion of the UP(P) carrier lipid disrupts PG synthesis. Indeed, this is the mechanism of action of bacitracin, which sequesters UPP. Depletion can also result from mutations or antibiotics that block other UP-dependent pathways, including lipo/oligosaccharides, WTA, and CPS synthesis. The impact of UP sequestration first emerged in studies of WTA. Mutations that block late stages of WTA synthesis led to cell death, whereas loss of the first enzyme in the pathway (TagO, Fig 2) leads to cells that are misshapen, but viable [54]. This insight led to the development of Targocil, an antibiotic that inhibits WTA export thereby depleting UP and triggering a shutdown of PG synthesis [55]. In *E. coli*, PG synthesis is also highly sensitive to conditions that lead to UP limitation [56,57].

Reuse it or lose it: PG recycling.

PG synthesis relies on the ordered insertion of newly synthesized glycan strands into the existing wall structure. While the details are still debated, active PG synthesis requires endopeptidases to cleave peptide crossbridges to make room for new strand insertion [58]. In addition, lytic transglycosylases degrade some of the existing PG structure to release muropeptides [58]. The generation and release of muropeptides during growth is substantial (Fig. 3), with up to ~50% of PG recycled each generation. In Gram-negative bacteria, where muropeptides are recaptured from the periplasm, recycling is efficient [59]. In Gram-positive bacteria there is significant loss of muropeptides to the environment. Studies in *S. aureus, B. subtilis,* and *Streptomyces coelicolor* suggest that aminosugars released from the cell wall may be recycled upon entry into stationary phase. However, the efficiency of recycling is low, with only 5–10% MurNAc recovery [60].

The Gram-positive cell wall additionally contains WTA, which can comprise up to 60% of the wall mass [61]. Synthesis of WTA, a sugar-phosphate copolymer, places a high demand on cellular phosphate reserves. In *B. subtilis*, the PhoPR regulatory system responds to phosphate limitation by repressing WTA synthesis and activating the synthesis of a substitute phosphate-free polymer, teichuronic acid (Fig 3) [62]. The cell may also recycle phosphate from existing WTA. PhoR activates expression of a WTA catabolic teichoicase (GlpQ) and phosphohydrolase (PhoD) (Fig 3) [63]. This phosphate scavenging mechanism can be used to selectively degrade the WTA of neighboring bacteria. For example, *S. aureus* produces a ribitol-phosphate WTA, and phosphate-limited cells express a teichoicase that releases glycerol-phosphate from the WTA of neighboring bacteria [64].

The virtue of charity: interspecies resource sharing.

The large metabolic demands associated with envelope synthesis may be reduced if precursors can be obtained from neighboring organisms. For example, the obligate intracellular pathogen *Rickettsia* relies on >50 host-derived metabolites for growth [65]. Among these, isoprenoid precursors are required for synthesis of the UP(P) carrier lipid [66]. As a result, treatment of mammalian cells with statins that reduce isoprenoid synthesis lead to a decreased proliferation and morphological defects in resident *Rickettsia* (Fig 4) [66].

Interspecies cross-feeding is also essential for the survival of *Tannerella forsythia*. This organism lacks GlmS, MurA, and MurB synthetic enzymes, and relies instead on scavenging

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of PG turnover products from its periodontal neighbors [59]. Co-habiting *Fusobacterium nucleatum* may act as a patron to provide muropeptides and perhaps LPS-derived sugars, and sialic acid may be scavenged from salivary mucins and glycoproteins in the oral mucosa [67]. It is likely that further analysis of complex microbial communities will reveal other examples of metabolically interdependent cells that share the burden of synthesizing cell envelope precursors.

Organisms in long-term associations may instead share the burden of enzyme production. The ability of an endosymbiont to transfer the metabolic burden of gene maintenance, transcription, and protein synthesis to its host is evolutionarily advantageous, assuming that the enzyme can be imported to fulfill its necessary function. The amoeba *Paulinella chromatophora*, for example, grows photosynthetically by virtue of a symbiotic association with a cyanobacterium that resides as a plastid (chromatophore) in the host cytosol [68]. This plastid retains a 1 Mb genome, but several metabolic pathways, including PG synthesis, require enzymes whose genes now reside in the amoeba. In the case of PG, the bacterial MurF enzyme for attaching D-Ala-D-Ala to the stem peptide is host encoded and must be imported for PG synthesis [68].

This type of gene transfer has been taken even further in a bacterium (*Moranella*) residing within another bacterium (*Tremblaya*), which in turn resides as a symbiont in an insect (mealybug) (Fig 4) [69]. Both bacterial symbionts have greatly reduced genomes, with only 400 protein-coding genes in *Moranella*, and 120 in *Tremblaya*. Although *Moranella* retains genes for most of the enzymes needed to assemble PG in the periplasm, it lacks those needed for the cytosolic steps leading to the lipid I precursor. These genes are in the insect genome, having been imported from multiple bacteria. Remarkably, to function in *Moranella*, these insect-synthesized proteins must cross five lipid bilayers (*Tremblaya* has three and *Moranella* two). The ability of *Moranella* to synthesize PG therefore reflects a shared burden of enzyme synthesis, with enzymes for the late stages of synthesis encoded by the endosymbiont and those for early stages imported from the insect host [69]. Given the ubiquity of reductive genome evolution in symbionts, it is likely that cell envelope synthesis is a shared burden in many other systems.

Conclusions.

Assembling a cell envelope imposes a large metabolic burden on cells. Cells have evolved a wide variety of mechanisms to economically regulate the flux of precursors needed for envelope biogenesis, often by controlling the activity of the enzymes at critical crossroads of metabolism. Situations that deplete required precursors, either through mutation, nutrient limitation, or the action of antimicrobial compounds, weaken the envelope and can lead to cell death. Cells may bypass precursor limitation by scavenging relevant nutrients from the environment or by cleaving cell surface structures from neighboring cells. Finally, we highlight examples where the metabolic burden is shared between intracellular bacteria and their hosts, either through provision of metabolic intermediates or by sharing the burden of enzyme synthesis.

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Highlights

- Bacteria are protected and shaped by membranes and a peptidoglycan wall
- Peptidoglycan is often linked to a polysaccharide capsule and teichoic acids
- Synthesis of the cell envelope requires a partitioning of shared metabolites
- The key branchpoint enzymes are regulated by multiple feedback mechanisms
- Key metabolites may be obtained from other bacteria or a eukaryotic host

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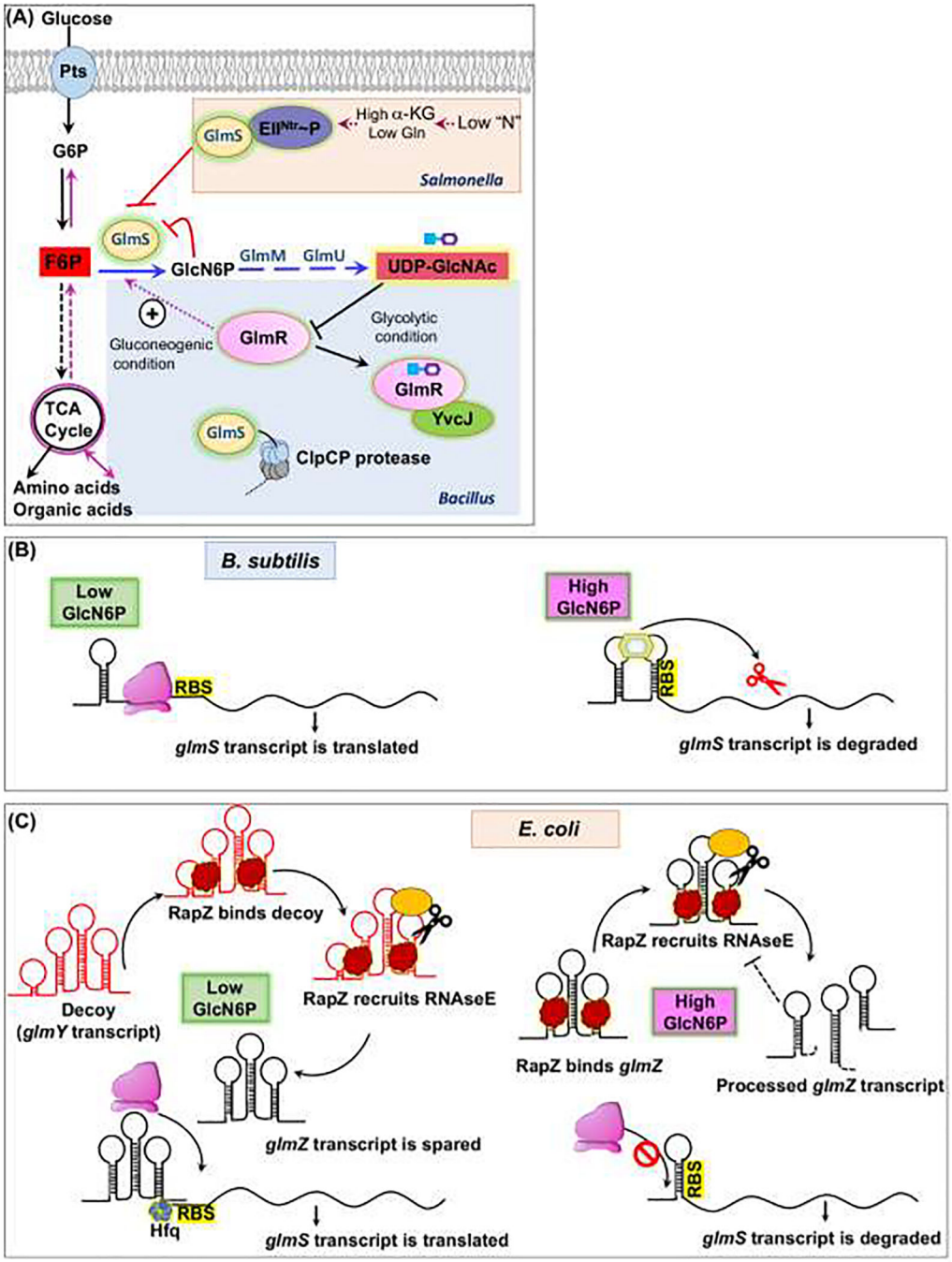

Figure 1. Regulation of the GlmS branchpoint enzyme.

A) Post-translational regulatory mechanisms. Glucose (Glc) is imported through a PTS and enters glycolysis where the branchpoint metabolite fructose-6-phosphate (F6P) is partitioned to aminosugar biosynthesis by GlmS. In *Salmonella* Typhimurium, GlmS is inhibited by the phosphorylated EII protein of the PTS^{NTR} system under conditions of nitrogen deficiency (sensed by a high α-ketoglutarate to glutamine ratio). In *B. subtilis*, GlmS is stimulated the regulator GlmR(YvcK), and this activation is inhibited by binding to the next key branchpoint metabolite UDP-GlcNAc (UDP=blue square; GlcNAc is purple hexagon),

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GlmR:UDP-GlcNAc binds to YvcJ, which reduced GlmS activity/ GlmS is also subject to ClpCP-dependent proteolysis. (B) Feedback regulation of GlmS translation. In *B. subtilis*, the *glmS* transcript includes a 5'-regulatory ribozyme that binds GlcN6P, resulting in cleavage and inactivation of the mRNA. (C) Translation of the *E. coli glmS* mRNA is activated for recognition by the ribosome (pink) when bound to the regulatory RNA *glmZ*. However, when GlcN6P is high the *glmZ* transcript is degraded by RNase E, which is recruited by RapZ. When GlcN6P is low, RapZ triggers expression of a decoy transcript (GlmY) that sequesters RapZ, and the GlmZ sRNA is spared to activate *glmS* translation.

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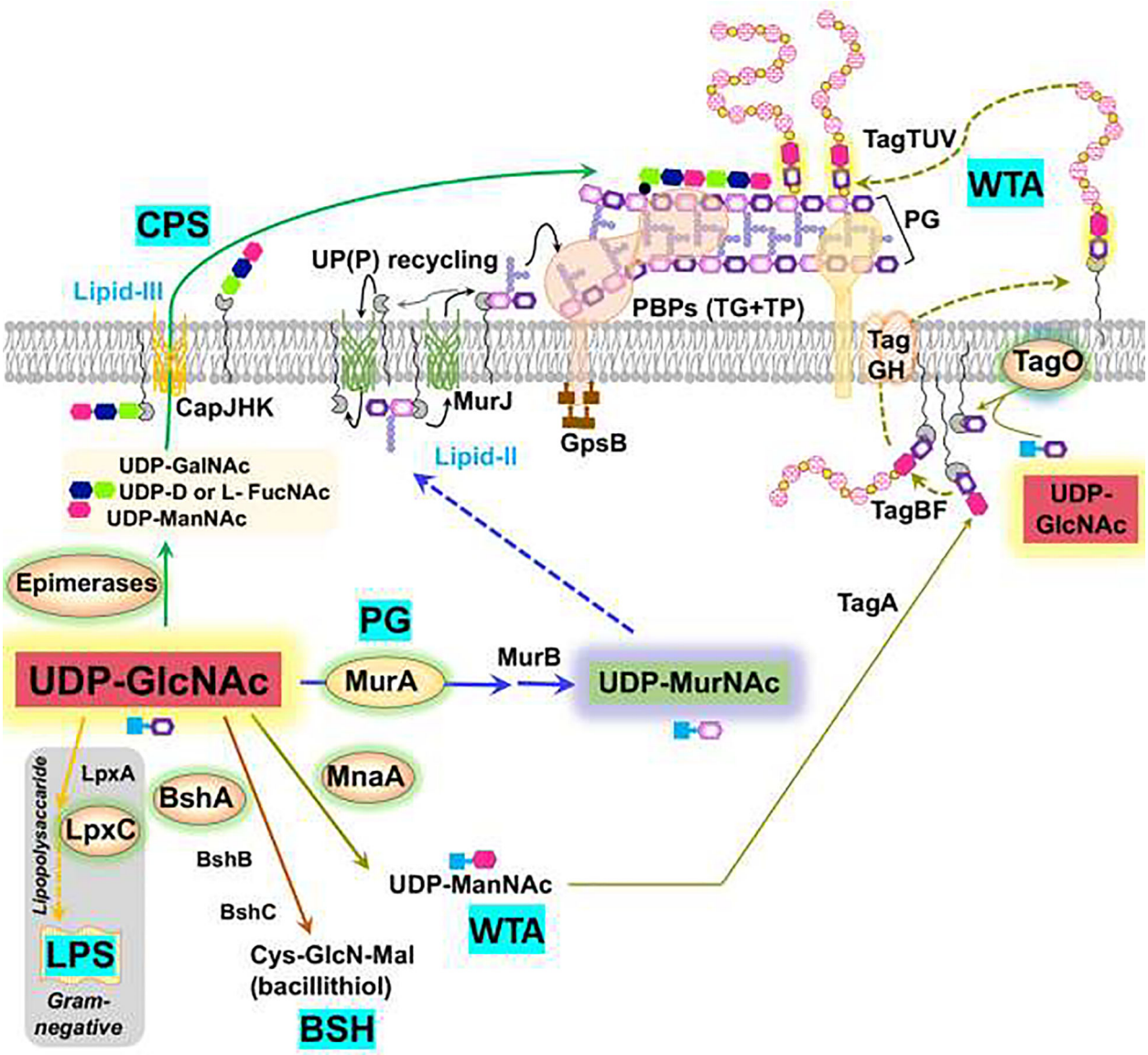

Figure 2. UDP-GlcNAc is a key branchpoint metabolite.

UDP-GlcNAc (red) is a shared precursor directed to peptidoglycan (PG) synthesis by MurA. PG synthesis relies on a UP-linked disaccharide-pentapeptide (lipid II), which is exported by MurJ so that penicillin-binding proteins (PBPs) and the Rod complex (elongasome) can catalyze the transglycosylation (TG) and transpeptidation (TP) reactions for PG synthesis. In Gram-positive bacteria, UDP-GlcNAc is additionally directed to wall teichoic acid (WTA) synthesis by MnaA (for UDP-ManNAc synthesis) and TagO (which couples GlcNAc to UP). WTA polymers are synthesized in the cytosol, flipped across the membrane by the TagGH complex, and covalently linked to PG (TagTUV enzymes). UDP-GlcNAc is also required for bacillithiol (BSH) synthesis, CPS synthesis and, in Gram-negative bacteria, for LPS biosynthesis. Branchpoint enzymes are highlighted with a yellow background.

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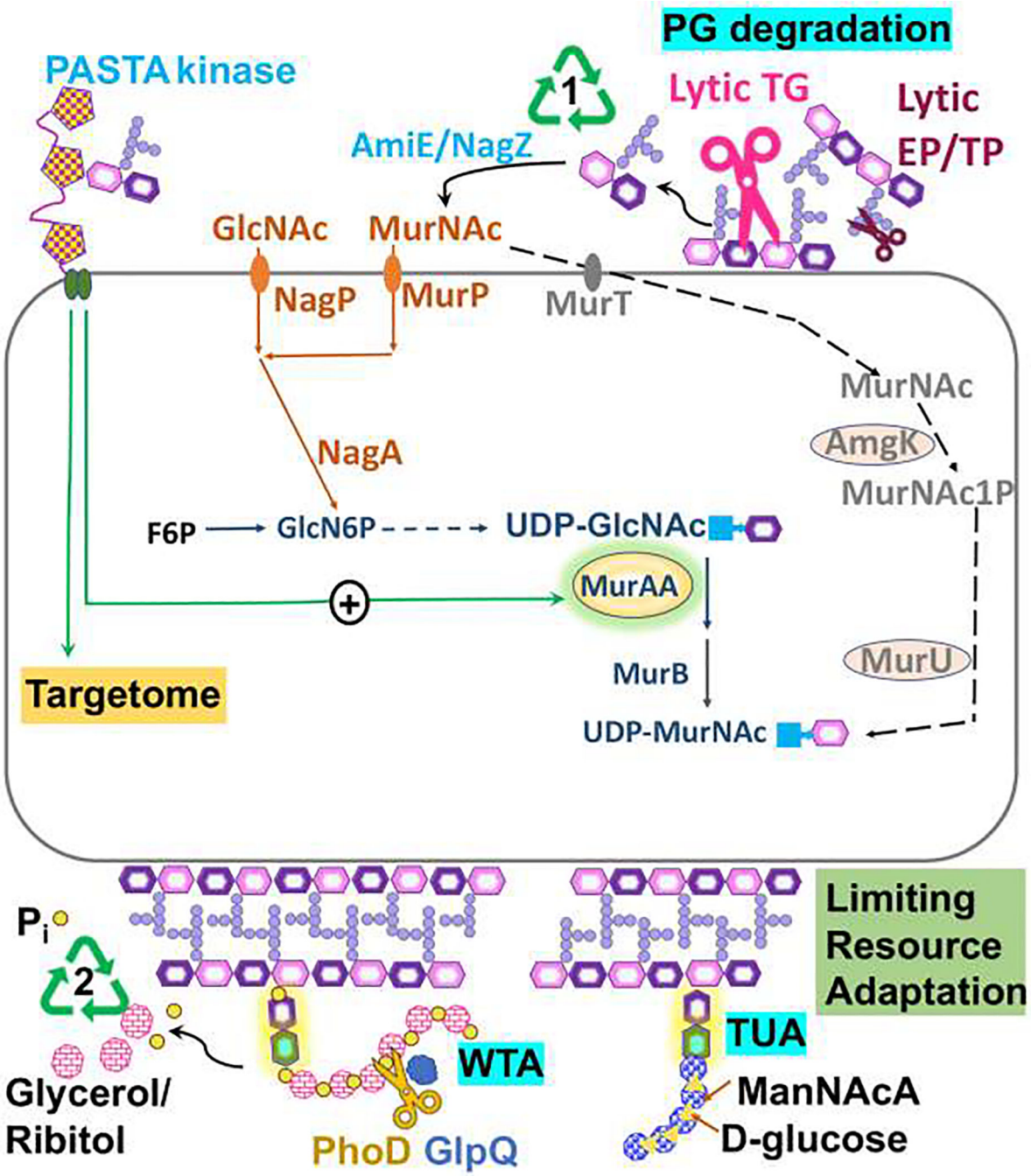

Figure 3. Degradation and recycling of the cell envelope.

Cell growth is accompanied by release of muropeptides generated by lytic transglycosylases (lytic TG) and endopeptidases/transpeptidases (lytic EP.TP) (1), which may be recycled. Recycling pathways involve degradation to release GlcNAc and MurNAc sugars and amino acids. Gram-negative bacteria may import muropeptodes and recycle intracellularly (not shown). In *B. subtilis* (orange), MurNAc and GlcNAc, are imported by PTS transporters MurP and NagP. The oral pathogen *Tannerella forsythia* is auxotrophic for MurNAc, which is imported by MurT and processed to generate UDPMurNAc (gray). Muropeptides can also

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activate some PASTA kinases, which phosphorylate many target proteins (targetome). In some systems, this can regulate the PG branchpoint enzyme MurA. (2) During phosphate limitation, Gram-positive bacteria may recycle WTA (glycerol-P copolymer) aided by the GlpQ and PhoD proteins. Secreted teichoicases may also cleave WTA off of nearby cells to scavenge phosphate. Phosphate limitation in *B. subtilis* triggers a switch from synthesis of a glycerol-P-based WTA to a functionally similar, but phosphate-free teichuronic acid polymer.

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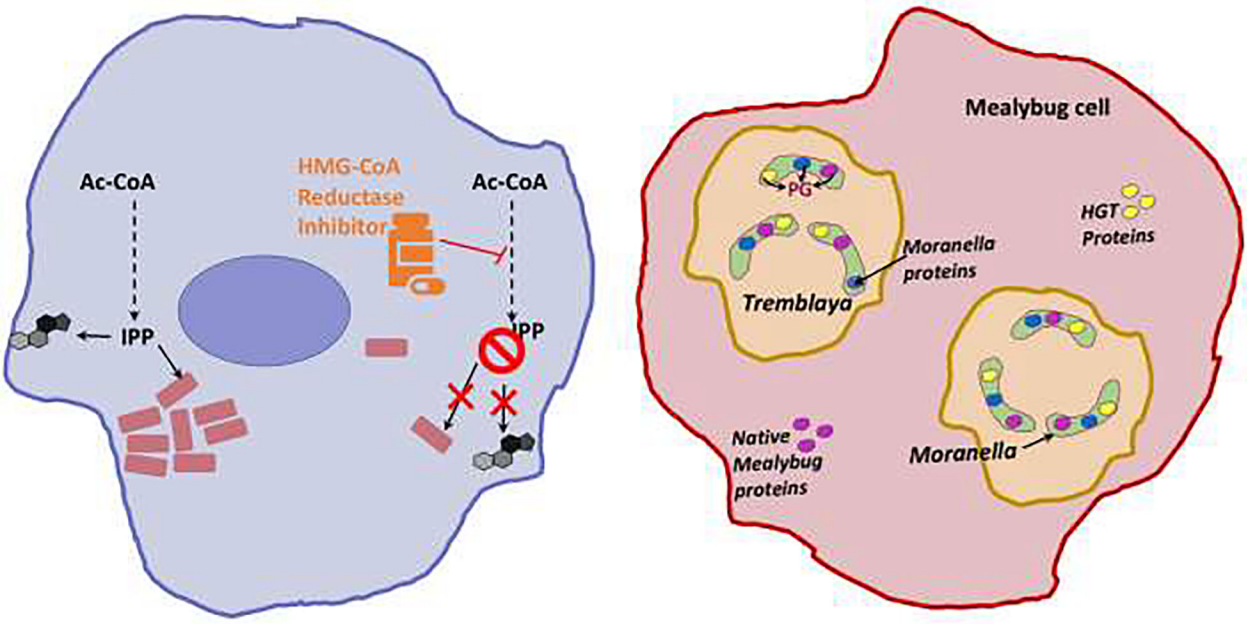

Figure 4. Resource sharing beyond kin.

Left: *Rickettsia* (pink rectangles) obtains isoprenoid precursors from its host. The inhibition of host HMG-CoA reductase restricts this metabolic crossfeeding and ultimately affects *Rickettsia* survival. **Right:** *Moranella* is snugly housed in *Tremblaya*, which in turn lives as an endosymbiont in its insect host, the mealybug *Planoccous citri* cells. These nested cells share the burden of PG synthesis for the diminutive *Moranella* cell. The initial enzymes for PG biosynthesis are imported from the Mealybug and include both housekeeping enzymes (Glm-S,U, and M-depicted in pink) and enzymes acquired by horizontal gene transfer from Bacteria (MurA-F, DdlB, DapF, MltB, AmiD- depicted in yellow). The cytosolic PG intermediate synthesized by these imported enzymes can then be assembled by later stage enzymes retained by *Moranella* (MraY-Pbp's- depicted in blue).