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Bacteria's different ways to recycle their own cell wall

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ABSTRACT

The ability to recover components of their own cell wall is a common feature of bacteria. This was initially recognized in the Gram-negative bacterium *Escherichia coli*, which recycles about half of the peptidoglycan of its cell wall during one cell doubling. Moreover, *E. coli* was shown to grow on peptidoglycan components provided as nutrients. A distinguished recycling enzyme of *E. coli* required for both, recovery of the cell wall sugar *N*-acetylmuramic acid (MurNAc) of the own cell wall and for growth on external MurNAc, is the MurNAc 6-phosphate (MurNAc 6P) lactyl ether hydrolase MurQ. We revealed however, that most Gram-negative bacteria lack a *murQ* ortholog and instead harbor a pathway, absent *in E. coli,* that channels MurNAc directly to peptidoglycan biosynthesis. This “anabolic recycling pathway" bypasses the initial steps of peptidoglycan *de novo* synthesis, including the target of the antibiotic fosfomycin, thus providing intrinsic resistance to the antibiotic. The Gram-negative oral pathogen *Tannerella forsythia* is auxotrophic for MurNAc and apparently depends on the anabolic recycling pathway to synthesize its own cell wall by scavenging cell wall debris of other bacteria. In contrast, Gram-positive bacteria lack the anabolic recycling genes, but mostly contain one or two *murQ* orthologs. Quantification of MurNAc 6P accumulation in *murQ* mutant cells by mass spectrometry allowed us to demonstrate for the first time that Gram-positive bacteria do recycle their own peptidoglycan. This had been questioned earlier, since peptidoglycan turnover products accumulate in the spent media of Gram-positives. We showed, that these fragments are recovered during nutrient limitation, which prolongs starvation survival of *Bacillus subtilis* and *Staphylococcus aureus*. Peptidoglycan recycling in these bacteria however differs, as the cell wall is either cleaved exhaustively and monosaccharide building blocks are taken up (*B. subtilis*) or disaccharides are released and recycled involving a novel phosphomuramidase (MupG; *S.aureus*). In *B. subtilis* also the teichoic acids, covalently bound to the peptidoglycan (wall teichoic acids; WTAs), are recycled. During phosphate limitation, the sn-glycerol-3-phosphate phosphodiesterase GlpQ specifically degrades WTAs of *B. subtilis*. In *S. aureus*, in contrast, GlpQ is used to scavenge external teichoic acid sources. Thus, although bacteria generally recover their own cell wall, they apparently apply distinct strategies for breakdown and reutilization of cell wall fragments. This review summarizes our work on this topic funded between 2011 and 2019 by the DFG within the collaborative research center SFB766.

1. Introduction: the dynamic bacterial cell wall

The cell wall of bacteria rests outside the cytoplasmic membrane and covers the entire cell. It contains the peptidoglycan (PGN), a cell-stabilizing and shape-maintaining macromolecule, building a sack-like structure, the murein or PGN sacculus. The sacculus structure of the bacterial PGN was first recognized in the 1960s in Tübingen by Wolfhard Weidel and his group (Weidel and Pelzer, 1964). They isolated pure PGN cell walls from *Escherichia coli* cells and demonstrated by electron microscopy that these maintain the size and rod-shape of the cells they were derived from (for an historical account see (Braun, 2015)). They also provided a first structural model of the PGN macromolecule, which is a mesh-like heteropolymer composed of long glycan strands of alternating *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) sugars cross-linked by short peptides ((Weidel and Pelzer, 1964); for recent reviews see (Litzinger and Mayer, 2010; Walter and Mayer, 2019)). The PGN sacculus is essential to protect bacteria from cell disruption and lysis due to high intracellular

Abbreviations: PGN, peptidoglycan; WTA, wall teichoic acids; LTA, lipoteichoic acids; MurNAc, *N*-acetylmuramic acid; GlcNAc, *N*-acetylglucosamine

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osmotic pressure (turgor), which can reach up to 5 atm in Gram-negative bacteria (containing a mostly single-layered PGN that is sandwiched between an inner and an outer membrane), and up to 50 atm in Gram-positive bacteria (containing a thick peptidoglycan wall but lacking an outer membrane) (Seltmann and Holst, 2002). In Gram-negative bacteria the PGN is connected to the outer membrane via Braun's lipoprotein, which is covalently linked with the PGN (Hantke and Braun, 1973). In Gram-positive bacteria, polyol-phosphate polymers are covalently attached to the PGN (wall teichoic acids; WTA) or are non-covalently enmeshed in the PGN network (lipoteichoic acids; LTA) (Brown et al., 2013; Percy and Gründling, 2014; Weidenmeier and Peschel, 2008).

The cell wall protects bacteria from adverse impacts of the environment and from disruption due to osmotic shock, thus it has to be sufficiently rigid. At the same time, it needs to be highly flexible, allowing bacteria to steadily adjust shape and mechanical properties of their cell wall during growth and differentiation. Thus, the PGN is constantly remodeled and degraded, involving large sets of potentially autolytic enzymes (autolysins), produced by the bacteria themselves, which potentially target every covalent bond connecting the building blocks within the PGN network (Vermassen et al., 2019). The peptide part of the PGN is cleaved by various amidases and peptidases, whereas the glycan part of the PGN is targeted by lytic transglycosylases, *N*-acetylmuramidases (or *N*-acetylmuraminidases) and *N*-acetylglucosaminidases (Fig. 1). As these cell wall-lytic enzymes are capable of destroying the own PGN mesh, the action of these enzymes must be delicately balanced in bacteria. Indeed, the action of autolysin is controlled by various mechanisms, including structural modifications within the PGN network, which is a distinctive process in bacteria. The Gram-positive pathogen *Staphylococcus aureus,* for example, protects itself from the action of lysozyme-like *N*-acetylmuramidases, cleaving the MurNAc-β-1,4-GlcNAc bond within the glycan backbone of the PGN, by *O*-acetylation of the MurNAc residues within the PGN (Bera et al., 2005). The bacterium therefore applies endogenous *N*-acetylglucosaminidases to degrade its own PGN during cell growth (Biswas et al., 2006; Oshida et al., 1995). *Bacillus subtilis* and many other bacteria in turn control cell wall cleavage via the action of *N*-acetylmuramidases and *N*-acetylglucosaminidases by partial de-*N*-acetylation of the amino sugar part of the PGN (Araki et al., 1980; Benachour et al., 2012; Kobayashi et al., 2012; Psylinakis et al., 2005). Control of PGN autolysis is also achieved by activation mechanisms that restrict the enzyme activity to certain regions, e.g. the inter septal region during cell separation (Meisner et al., 2013; Yang et al., 2011). This allows cell wall degradation within regions prone to decay and leaves portions intact that are required for cell wall stability.

Cleavage of the PGN by endogenous autolytic enzymes releases cell wall-derived fragments (cell wall turnover). In Gram-positive bacteria large amounts of these fragments are found in the growth medium, as these lack an outer membrane as permeability barrier (Reith and Mayer, 2011). This had been the reason why the capability of Gram-positive bacteria to recycle their cell wall had been questioned earlier (Reith and Mayer, 2011). In Gram-negative bacteria, the outer membrane restricts diffusion of larger molecules and cell wall-derived fragments are mostly trapped within the periplasm, from which they are efficiently recovered (cell wall recycling). Cell wall recycling was initially recognized in *E. coli* (Goodell, 1985). This organism releases only small amounts of cell wall-derived peptides into the medium, the major part of the PGN turnover products however are recovered (Goodell, 1985; Goodell and Schwarz, 1985). In *E. coli* and other Gram-negative bacteria, the continuous cleavage of the PGN within the periplasm involves lytic transglycosylases, which generate 1,6-anhydro-MurNAc (anhMurNAc)-containing PGN fragments (Dik et al., 2017b) (Fig. 1). These anhydromuropeptides (GlcNAc-anhMurNAc-peptides) are the major PGN recycling products of Gram-negative bacteria (Park and Uehara, 2008). It was estimated that as much as half of the PGN is recycled during one cell doubling in *E. coli*, thus PGN recycling represents a major salvage pathway of bacteria. Recycling of anhydromuropeptides had attracted particular attention due to the connection with β-lactam resistance in Enterobacteriales and Pseudomonales (Bertsche et al., 2015; Dik et al., 2018; Fisher and Mobashery, 2014; Jacobs et al., 1997; Mark et al., 2011). These bacteria are able to sense the relative amounts of the PGN precursor UDP-MurNAc-pentapeptide

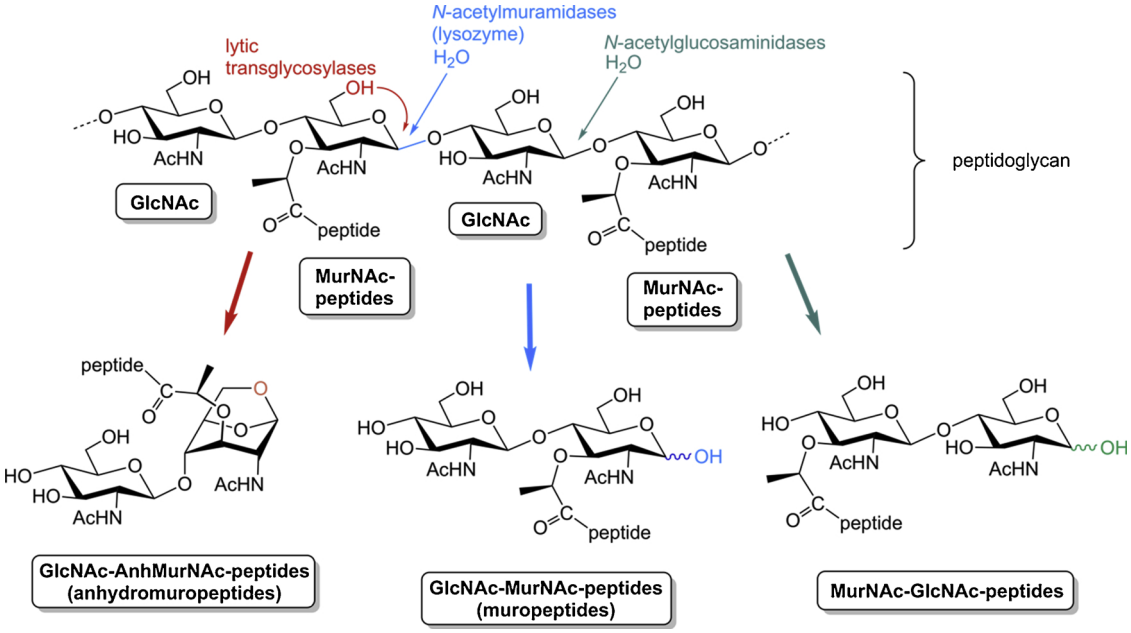

Fig. 1. The glycan backbone of the PGN can be cleaved by lytic transglycosylases (LTs), *N*-acetylmuramidases, and *N*-acetylglucosaminidases. LTs and *N*-acetylmuramidases catalyze cleavage of the same glycosidic bond (MurNAc-β-1,4-GlcNAc). However, only the *N*-acetyl-muramidases catalyze hydrolysis this bond, generating GlcNAc-MurNAc-peptides (muropeptides), whereas the LTs catalyzes non-hydrolytic cleavage, generating 1,6-anhydroMurNAc (AnhMurNAc)-containing fragments (anhydromuropeptides). Endo-acting *N*-acetylglucosaminidases catalyze the hydrolysis of the other glycosidic bond (GlcNAc-β-1,4-MurNAc), thus generating distinct MurNAc-GlcNAc-peptides. It should be noted here that recently LTs have been identified, which are also able to generate 1,6-anhydro-GlcNAc-containing fragments (Williams et al., 2018).

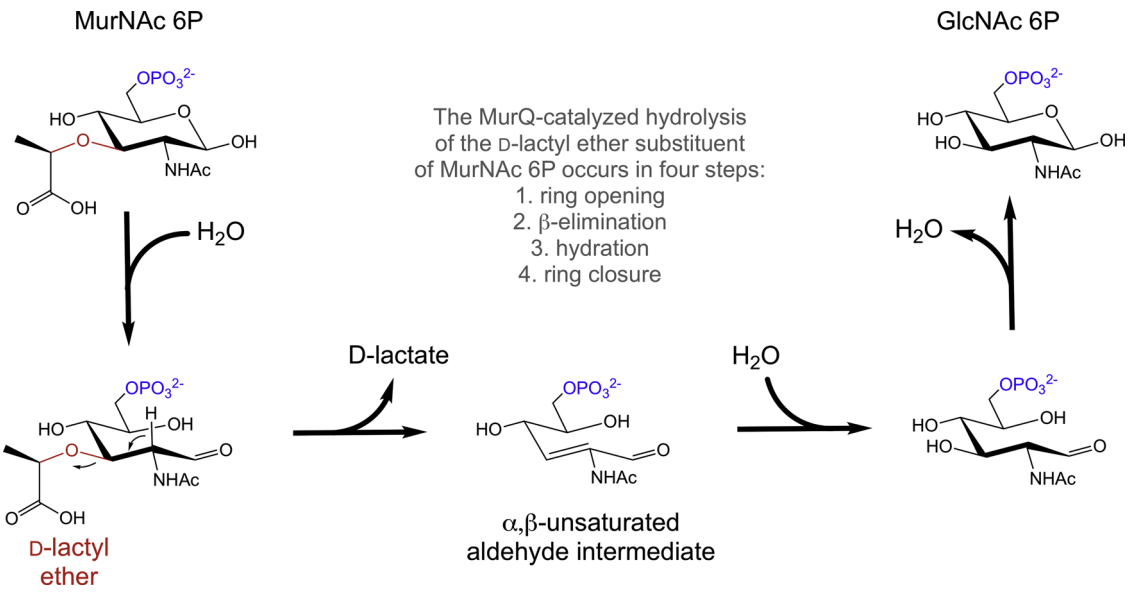

Fig. 2. The etherase MurQ is a distinguished cell wall recycling enzyme, first identified in *E. coli*. It catalyzed the hydrolysis of the D-lactyl ether substituent of MurNAc 6P by an β-elimination/hydration mechanism and via formation of an α,β-unsaturated aldehyde intermediate, finally yielding GlcNAc 6P.

and the cell wall turnover product anhydroMurNAc-pentapeptide, thereby monitoring the status of the cell wall and eventually inducing the expression of chromosomally encoded AmpC-type β-lactamase (Dik et al., 2017a; Vadlamani et al., 2015).

2. The etherase MurQ and PGN recycling in *E. coli*

A dedicated PGN recycling enzyme had been identified earlier in our group, which converts MurNAc 6-phosphate (MurNAc 6P) to GlcNAc 6-phosphate (GlcNAc 6P) (Jaeger et al., 2005). The enzyme acts as a unique MurNAc 6P lactyl ether hydrolase (named etherase MurQ) that cleaves off the D-lactic acid side chain from MurNAc 6P by a β-elimination/hydration mechanism (Hadi et al., 2008; Jaeger et al., 2005; Jaeger and Mayer, 2008a) (Fig. 2). MurQ had been initially characterized as an enzyme required for growth of *E. coli* on MurNAc as the sole source of carbon and energy, together with the MurNAc-PTS transporter MurP (Dahl et al., 2004; Jaeger et al., 2005; Jaeger and Mayer, 2008b). Later on, it was shown that MurQ is also necessary for the recycling of anhMurNAc (Uehara et al., 2006). Anhydromuropeptides, generated by lytic transglycosylases, are imported into the cell via AmpG permease and AnhMurNAc is released together with GlcNAc and cell wall-peptides by the concerted action of dedicated PGN recycling enzymes: the *N*-acetylglucosaminidase NagZ, the AnhMurNAc-L-alanine amidase AmpD, and the L,D-carboxypeptidase LdcA (Park and Uehara, 2008) (Fig. 3). AnhMurNAc is further phosphorylated by a specific AnhMurNAc kinase (AnmK) yielding MurNAc 6P (Uehara et al., 2005), GlcNAc is converted to GlcNAc 6P by the GlcNAc kinase NagK (Park and Uehara, 2008) and MurQ converts MurNAc 6P to GlcNAc 6P (Jaeger et al., 2005). Finally, GlcNAc 6P enters the known catabolic pathway of GlcNAc or is used for PGN re-synthesis (Park and Uehara, 2008; White, 1968). The peptide portion of the PGN can be catabolized after cleavage by amidases/peptidases (during amino acid starvation), but normally the intact murein tripeptide L-Ala-iso-D-Glu-mDAP is added to UDP-MurNAc by the Mpl ligase, yielding UDP-MurNAc-tripeptide, thereby substituting three amino acid ligase enzymes (MurCDE) of the PGN *de novo* biosynthesis (Park and Uehara, 2008). Notably, the L,D-carboxypeptidase LdcA is required to trim cell wall-derived tetra- and pentapeptides to tripeptides. If this enzyme is missing, *E. coli* cell lyse in the onset of stationary phase (Templin et al., 1999), presumably because accumulation of tetrapeptides leads to the formation of "wrong" UDP-MurNAc-tetrapeptide precursors that do not allow the PGN to get normally crosslinked by D,D-transpeptidation.

3. An anabolic recycling route identified in *Pseudomonas* sp

We recognized that the MurNAc 6P etherase (MurQ) unexpectedly is absent in many Gram-negative bacteria (Jaeger and Mayer, 2008a). Most *Pseudomonas* sp. for example, lack MurQ despite possessing all upstream recycling enzymes, e.g. AmpG, AmpD, NagZ, and AnmK. They also contain the recycling route that channels murein tripeptide to PGN biosynthesis (LdcA, Mpl). In *Pseudomonas putida*, all enzymes required for the catabolism of cell wall-derived amino sugars are missing, including MurP, MurQ, NagK and also NagA and NagB. We hypothesized that in organisms that lack MurQ the amino sugars of the PGN cell wall might be channeled directly to PGN biosynthesis, by a so far unknown route, analogous to the tripeptide recycling via Mpl. We further argued that a hypothetical PGN recycling bypass to UDP-MurNAc should affect fosfomycin resistance in these organisms (Gisin et al., 2013). Fosfomycin is a broad spectrum antibiotic that targets the enzyme MurA catalyzing first step of PGN synthesis towards the formation of uridine diphosphate *N*-acetylmuramic acid (UDP-MurNAc) (Fig. 4). Since about half of the PGN is recycled in one generation, this should massively contribute to the UDP-MurNAc pool, and should thus affect fosfomycin susceptibility. Indeed, when we tested known *Pseudomonas* recycling mutants (*ampG, nagZ, ampD*), they were severely affected in fosfomycin susceptibility (Borisova et al., 2014; Gisin et al., 2013). We used this test, to screen putative candidate genes involved in an UDP-MurNAc biosynthetic (anabolic) recycling metabolism. A nucleotidyl transferase similar to transferase domain of GlmS (the enzyme converting glucosamine 6P to UDP-MurNAc) was identified as MurNAc α-1-phosphate uridylyltransferase (MurU) and the preceding enzyme was found to be an anomeric kinase that phosphorylates MurNAc as well as GlcNAc at the α-1-position (AmgK, for anomeric MurNAc/GlcNAc kinase) (Gisin et al., 2013; Renner-Schneck et al., 2015). Simultaneously, in the group of Tom Bernhardt and in our group the last enzyme of the anomeric recycling route was identified, a specific MurNAc 6P phosphatase named MupP (Borisova et al., 2017; Fumeaux and Bernhardt, 2017). Thus, the complete "anabolic PGN recycling pathway" of *Pseudomonas* sp. includes the phosphorylation of anhMurNAc by AnmK, dephosphorylation by MupP, re-phosphorylation at the anomeric position with AmgK and the uridylyl transfer reaction catalyzed by MurU, yielding UDP-MurNAc (Fig. 4). The anomeric kinase AmgK is also able

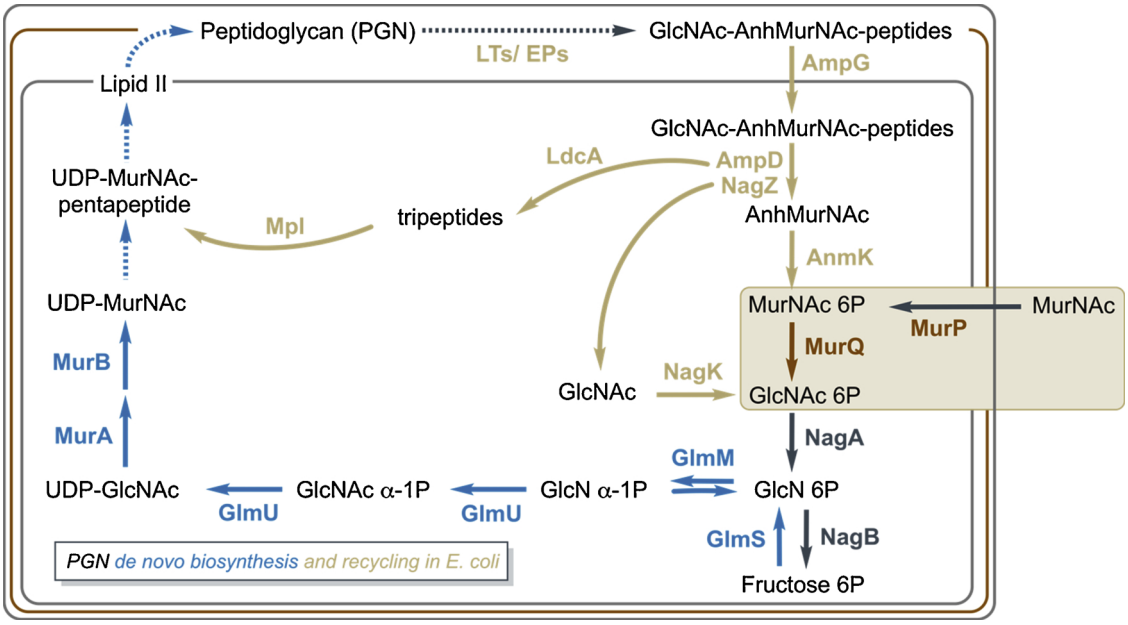

Fig. 3. Overview of the PGN recycling and MurNAc catabolic pathways of *E. coli.* PGN turnover in *E. coli* involves lytic transglycosylases (LTs) and endopeptidases (EPs) which generate GlcNAc-AnhMurNAc-peptides (anhydromuropeptides) in the periplasm. These are taken up by the AmpG permease and are then proceed in the cytoplasm by dedicated recycling enzymes: the *N*-acetyl-glucosaminidase NagZ, the AnhMurNAc-L-alanine amidase AmpD and the L,D-carboxypeptidase LdcA. Tripeptides are channeled into the PGN biosynthesis or are catabolized and the amino sugars AnhMurNAc and GlcNAc are phosphorylated by specific kinases (AnmK, NagK) and further catabolized via NagA/NagB or used for PGN resynthesis. The MurQ etherase is required for both, recycling of anhydromuropeptides as well as for growth on external MurNAc, which is imported and concomitantly phosphorylated via MurP.

to phosphorylate GlcNAc, yielding GlcNAc α-1-phosphate, and this product is accepted as a substrate by GlmU (bifunctional *N*-acetylglucosamine 1-phosphate uridylyltransferase). Thus both amino sugars of the PGN are recycled and channeled into the PGN biosynthesis.

The fosfomycin sensitivity phenotype of recycling mutants could be rescued, not only by complementation with the respective recycling genes from *Pseudomonas* sp., but also by expressing distantly related orthologs from *Neisseria menigititis* (β-Proteobacterium), *Caulobacter crescentus* (α-Proteobacterium), or *Tannerella forsythia* (Bacteroidetes) (Gisin et al., 2013); Hottmann, unpublished). This revealed that distantly related recycling genes are functional and, thus, the anabolic recycling route constitutes a common pathway within Gram-negative bacteria. The distribution of the different recycling enzymes within the bacterial phylogeny is shown in Fig. 5. The major conclusions drawn from this tree are: i) Gram-negative bacteria mostly contain the anabolic recycling route except for *E. coli* and related Enterobacteriales,

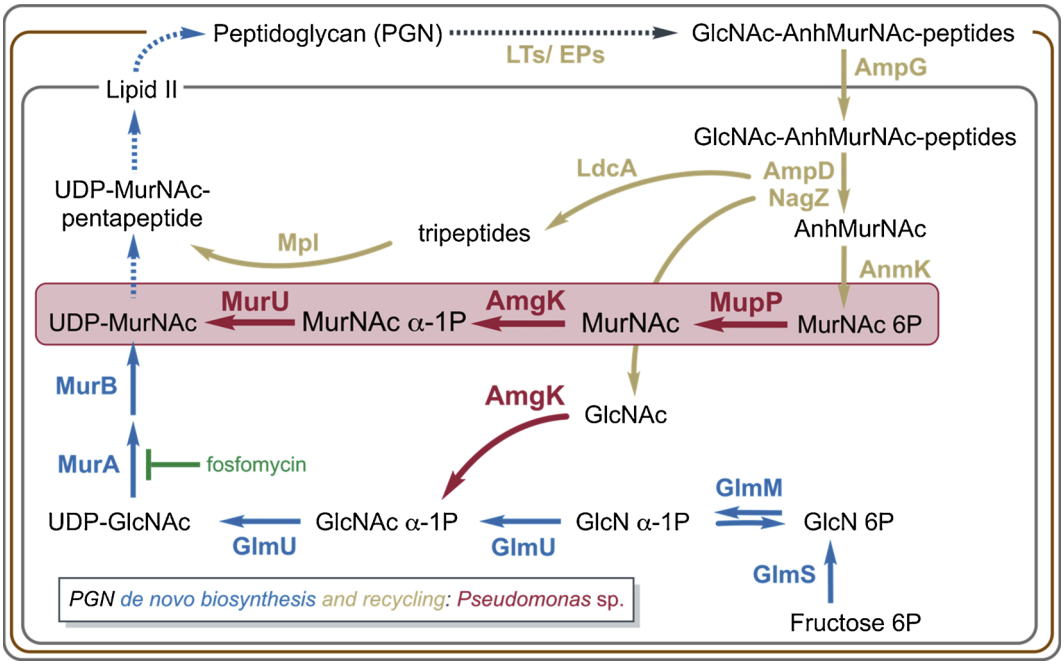

Fig. 4. Overview of the PGN recycling in *Pseudomonas putida.* The MurQ enzyme and most other enzymes involved in amino sugar recycling and catabolism are missing in *Pseudomonas* sp. Instead these bacteria harbor an "anabolic recycling pathway" (MupP, AmgK, MurU) that channels MurNAc 6P into the PGN biosynthesis pathway on the level of UDP-MurNAc, and GlcNAc to UDP-GlcNAc (AmgK, GlmU).

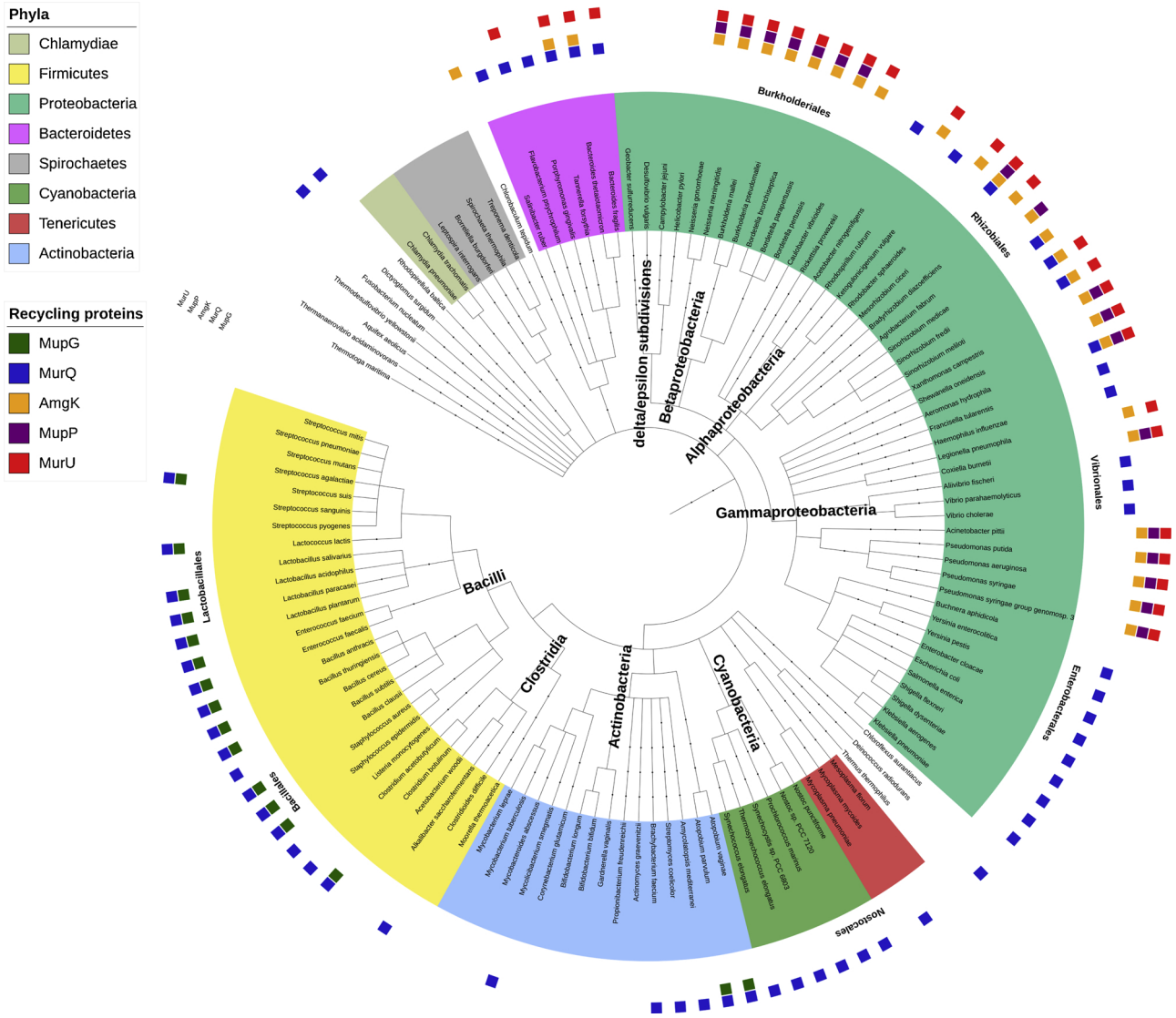

Fig. 5. Phylogenetic tree of representative bacterial species showing the distribution of PGN recycling-associated proteins in bacterial taxa. MurQ (blue; represents the MurQ pathway known from *E. coli* and *B. subtilis*) and MupG (green; see chapter entitled "Peptidoglycan recycling in *Staphylococcus aureus*" for description of MupG function); AmgK (orange), MupP (purple) and MurU (red), constituting the anabolic recycling pathway known from *Pseudomonas* sp. The tree was built on the taxonomy according to NCBI and BLAST searches for proteins identified in (Fumeaux and Bernhardt, 2017; Gisin et al., 2013). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Vibrionales and some others. Only few bacteria, mostly Bacteroidetes and Rhizobiales sp., contain both the catabolic recycling pathway (MurQ) and the anabolic recycling pathway (MupP/AmgK/MurU). The latter pathway, however, is absent in Cyanobacteria and in all Gram-positives, which generally contain one or two MurQ orthologs (Fig. 5). Intriguingly, some bacterial sp. (e.g. *Streptococcus* sp., as well as δ− and ε-Proteobacteria) apparently lack both pathways and it remains to be investigated, whether these are not able to recycle their cell wall (as had been suggested (Boersma et al., 2015)) or they apply different strategies to recover their cell wall components.

4. MurNAc recycling and scavenging in *Tannerella forsythia*, a MurNAc auxotrophic bacterium

Only few bacteria, e.g. some Bacteroidetes species, contain both the MurQ and the AmgK/MurU pathway. One such example is the oral pathogen *Tannerella forsythia.* This bacterium is phylogenetically related to *Bacteroides* species, but was classified based on 16S rRNA-analyses into its own genus *Tannerella* within the *Porphyromonadacea* (Sakamoto et al., 2002) (cf. Fig. 5). We are currently exploring the PGN metabolism in *T. forsythia*. Its natural habitat is the oral cavity where it lives as in a consortium together with *P. gingivalis* and *T. denticola*, called the “red complex consortium”. This complex is known to cause severe forms of periodontitis (Holt and Ebersole, 2005). The most intriguing observation regarding *T. forsythia* is that this organism has a demand for MurNAc if grown in axenic culture (Wyss, 1989). The observed MurNAc auxotrophy of *T. forsythia* appeared consequential after genome analysis (Friedrich et al., 2015): *T. forsythia* lacks *murA/murB* and *glmS/glm*U orthologs (see Fig. 6), which are generally essential for PGN *de novo* biosynthesis in other bacteria. As *T. forsythia* apparently is unable to synthesize MurNAc on its own, it has to acquire this amino sugar from the environment. Indeed *T. forsythia* was shown to grow in co-culture with different bacteria (Sharma et al., 2005). Many bacteria use a phosphotransferase system (PTS) transporter (MurP in *E. coli*) for the uptake of MurNAc (Dahl et al., 2004). These transporters phosphorylate MurNAc by the means of a phosphorylation cascade concomitant with the uptake (Deutscher et al., 2006). Thorough examination revealed that *T. forsythia* entirely lacks PTS transporters.

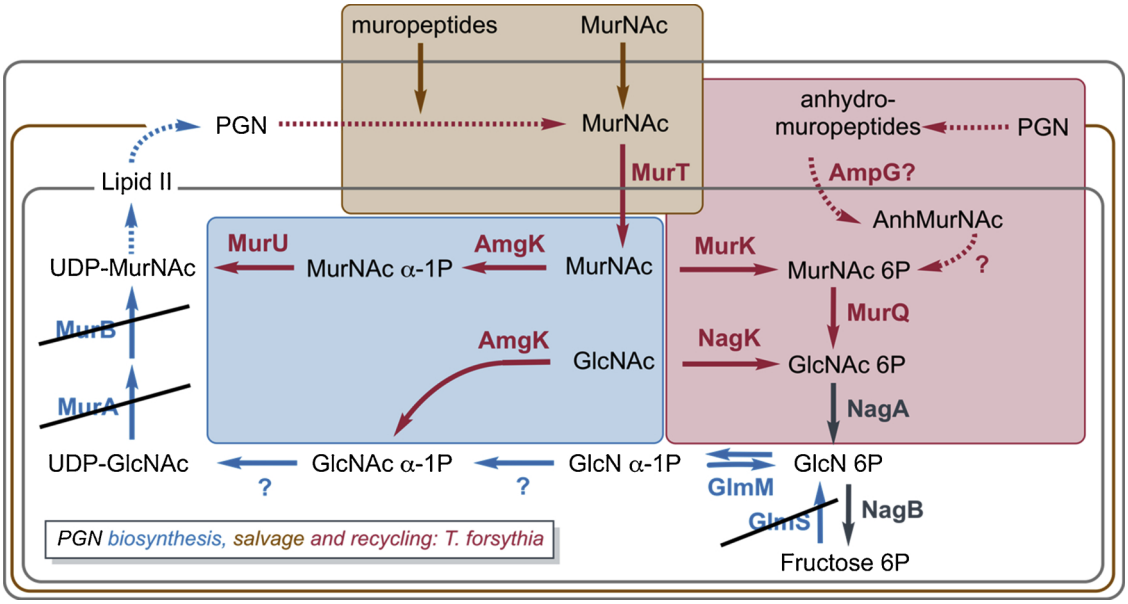

Fig. 6. Overview of the PGN recycling pathways of the oral pathogen *Tannerella forsythia*, which harbors both, the *E. coli* MurQ and the *Pseudomonas AmgK-MurU* pathways. *T. forsythia* lacks generally essential enzymes of the PGN *de-novo* biosynthesis pathway (blue), thus is auxotrophic for MurNAc. MurNAc uptake is mediated by a non-PTS tranporter (MurT) and the sugar is then either phosphorylated at the C6 hydroxyl group (MurK), thereby is prone for energy metabolism, or phosphorylated at the anomeric position (AmgK), leading to UDP-MurNAc. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

However, this organism carries a putative *murQ* orthologue (*Tanf_08385*) on its chromosome, which encodes a functional MurNAc 6P lactyl ether hydrolase named Tf_MurQ (Ruscitto et al., 2016). Two other genes are located upstream of *murQ* on the *T. forsythia* genome: a membrane protein (*Tanf_08375*) and a putative kinase (*Tanf_08380*). The membrane protein was identified as a PTS-independent MurNAc transporter named Tf_MurT, constituting a novel transporter family conserved within the Bacteroidetes (Ruscitto et al., 2016). The gene upstream of *murQ* subsequently was identified as a gene encoding an amino sugar kinase (Tf_MurK) that specifically phosphorylates MurNAc at the position C6, thereby generating MurNAc 6P, the substrate for Tf_MurQ (Hottmann et al., 2018). Biochemical characterization revealed that the Tf_MurK kinase exhibits exclusive specificity for MurNAc, thereby distinguishes from the GlcNAc/MurNAc-kinase of *Clostridium acetobutylicum* (Reith et al., 2011). In *T. forsythia* the Tf_MurT/MurK/MurQ pathway displays a MurNAc catabolic route generating GlcNAc 6P, which is further shuttled into glycolysis (Hottmann et al., 2018). Surprisingly, however, the *Tf_murK* deletion mutant was found to accumulate the PGN precursor molecule UDP-MurNAc-pentapeptide in stationary growth phase and cells showed a growth advantage in MurNAc-limited medium (Hottmann et al., 2018). These results indicate that in this organism, MurNAc may be shuttled into PGN biosynthesis via an anabolic pathway. Genome analysis revealed that orthologues of *amgK* and *murU* are indeed present in *T. forsythia*. Likely the AmgK/MurU pathway replaces the MurA/B pathway and represents the principle route for PGN synthesis in this organism (Fig. 6).

5. Peptidoglycan recycling in *Bacillus subtilis*

As mentioned above, an ortholog encoding the recycling enzyme MurQ is found on the genome of *B. subtilis* (48% amino acid sequence identity with *E. coli* MurQ). It is organized in an operon together with orthologs of *murR*, encoding a transcriptional MurNAc 6P-sensitive repressor, and *murP*, encoding a phosphotransferase system (PTS) transporter for MurNAc (amino acid sequence identities of 27% and 38%, respectively) (Dahl et al., 2004; Jaeger and Mayer, 2008b). Using high performance liquid chromatography/mass spectrometry (HPLC-MS), we could show that MurNAc 6P accumulates in *B. subtilis* Δ*murQ* cells grown in nutrient-rich medium (Borisova et al., 2016; Borisova and Mayer, 2017). The corresponding wild-type strain revealed no accumulation of MurNAc 6P, and also no accumulation was observed in a mutant lacking the whole operon, *murQ-murR-murP* (Borisova et al., 2016). This led to the conclusion that recycling of the MurNAc portion of the PGN takes place in Gram-positives and relies on a functional MurQ etherase. In addition, we showed that uptake and concomitant phosphorylation of MurNAc occurs via MurP (Borisova et al., 2016). The absence of MurNAc 6P accumulation in the operon mutant further indicates that MurP is the only transporter for MurNAc in *B. subtilis*. MurNAc 6P accumulated only in small amounts during exponential growth phase, but increased significantly in transition phase, and reached a maximum in stationary phase (Borisova et al., 2016; Unsleber et al., 2017). Thus, apparently PGN recycling in *B. subtilis* proceeds differently from *E. coli*, where recycling occurs continuously during exponential growth phase (Borisova et al., 2016; Jaeger and Mayer, 2008b). MurNAc 6P accumulation strongly increased when the growth medium was supplemented with MurNAc. Addition of MurNAc however did not sustain growth neither of wild-type nor of Δ*murQ* mutant cells. However, in late stationary phase, cultures of *B. subtilis* wild-type cells remained higher optical density when grown in a medium supplemented with MurNAc as compared to cultures of mutant cells or cultures of cells grown without MurNAc. This led to the assumption that MurNAc might have an impact on cell survival (Borisova et al., 2016). Indeed, higher numbers of colony forming units were observed in late stationary phase in wild-type cells grown with MurNAc, whereas Δ*murQ* cells showed a dramatic decrease in viability (Borisova et al., 2016). A survival benefit, albeit much weaker, was also observed when wild-type versus mutant cells were grown in nutrient rich-medium without MurNAc (Borisova et al., 2016). The lower viability of Δ*murQ* cells is likely due to increased cell lysis in the stationary phase. *B. subtilis* is known to lyse under nutrient-depletion conditions involving autolytic enzymes by mechanisms not completely understood (Jolliffe et al., 1981; Lewis, 2000; Smith et al., 2000). Usually autolysins are strictly regulated, however during nutrient limitation they apparently become deregulated. There is evidence that the collapse of the proton motive force (PMF) is responsible for the activation of autolysins, which

involves the proton buffering capacity of teichoic acids (Calamita and Doyle, 2002; Calamita et al., 2001; Jolliffe et al., 1981). In the onset of sporulation, *B. subtilis* undergoes cell lysis by a mechanism named cannibalism, which is the feeding of a starving subpopulation on sibling cells (Gonzalez-Pastor et al., 2003). In a heterogeneous cell population, some cells recognize nutrient limitation earlier than others. These cells activate the global regulator Spo0A by phosphorylation (Spo0A-P) (Chastanet et al., 2010; Chung et al., 1994). Spo0A-P is responsible for the expression of cannibalism toxins, sporulation delay factor (SDP) and sporulation killing factor (SKF), as well as their cognate immunity proteins (Ellermeier et al., 2006; Gonzalez-Pastor, 2011). Cannibalistic cells attack prey cells which have not sensed nutrient limitation, and hence have not activated Spo0A. Activity of the toxins SDP and SKF on prey cells leads to a collapse of the PMF and in a second consequence leads to the activation of autolysins, and thereby cell lysis (Lamsa et al., 2012). Lysed cells release nutrients including peptidoglycan fragments due to autolysin action, on which cannibalistic cells can feed on. *B. subtilis* cleaves peptidoglycan by endo-acting (Smith et al., 2000) as well as exo-acting hydrolases (*N*-acetylglucosaminidase NagZ and MurNAc-L-alanine amidase AmiE) (Litzinger et al., 2010). Thereby released cell wall sugars, MurNAc and GlcNAc, are taken up into the cytoplasm and are concomitantly phosphorylated by PTS transporters (Fig. 7). The MurQ etherase converts MurNAc 6P to GlcNAc 6P, which is deacetylated by NagA, and the product glucosamine 6P (GlcN 6P) either enters glycolysis or is used for PGN re-synthesis. Also the peptide portion of the PGN is recovered, which is however less well investigated (Amoroso et al., 2012). Thus, recovery of prey cell peptidoglycan might help cannibalistic cells to enhance survival during nutrient limitation and to delay sporulation (Lamsa et al., 2012).

6. Peptidoglycan recycling in *Staphylococcus aureus*

Staphylococcus aureus is a spherical non-sporulating Gram-positive bacterium, belonging to the phylum Firmicutes. It is usually part of the human microbiota, but methicillin-resistant *S. aureus* (MRSA) can also cause life-threatening infections due to multiple drug resistance (Hiramatsu et al., 2014; Tong et al., 2015). *S. aureus* protects itself from the action of lysozyme-like *N*-acetyl muramidases by *O*-acetylation of the C6 hydroxyl group of MurNAc residues within the PGN (Bera et al., 2005; Moynihan et al., 2014). In consequence, the bacterium cleaves its own PGN network using endo-acting *N*-acetylglucosaminidases, instead of *N*-acetylmuramidases, which are not affected by MurNAc *O*-acetylation. *S. aureus* processes, besides other *N*-acetylglucosaminidases, the enzyme Atl, which is the major autolysin of the organism (Bose et al., 2012; Oshida et al., 1995; Wheeler et al., 2015; Yamada et al., 1996). Atl is a bifunctional *N*-acetylmuramyl-L-alanine amidase/endo-*N*-acetylglucosaminidase, that is affected by binding to cell wall structures and releases MurNAc-GlcNAc disaccharide and peptides from the cell wall (Biswas et al., 2006; Götz et al., 2014; Oshida et al., 1995; Schlag et al., 2010). Atl is proteolytically processed and occurs in a cell surface-bound as well as a soluble form in the culture supernatant (Komatsuzawa et al., 1997; Yamada et al., 1996).

Alike *E. coli* and *B. subtilis*, *S. aureus* is able to recover extracellular MurNAc via the MurQ pathway (Borisova et al., 2016). However, the Atl autolysin as well as other *N*-acetylglucosaminidases of *S. aureus* generate MurNAc-GlcNAc disaccharides instead of MurNAc. The *S. aureus* recycling operon, encodes genes for the MurQ etherase, the PTS transporter MurP (SaMurP) and the MurR-like transcriptional regulator SaMurR. In addition, the operon contains a gene of unknown function downstream of *murQ*. We recently showed that this gene (*SAUSA300_0192*) encodes a 6-phospho-*N*-acetylmuramidase (named MupG), which cleaves the phosphorylated disaccharide MurNAc 6P-GlcNAc yielding MurNAc 6P and GlcNAc (Kluj et al., 2018). Accordingly, MurNAc 6P-GlcNAc accumulates in the *S. aureus* USA300 Δ*mupG* mutant. As the disaccharide MurNAc-GlcNAc accumulates in the culture supernatant of a *S. aureus ΔmurP* mutant, which is the main turnover product in *S. aureus* generated by Atl, the natural substrate of the PTS transporter is the disaccharide MurNAc-GlcNAc and not MurNAc. *Sa*MurP is able to transport and phosphorylate both the disaccharide as well as MurNAc, however, MurNAc is a fortuitous substrate and not a natural product of PGN degradation in this organism. By fragmentation MS, the product of phosphorylation by SaMurP and the respective substrate for MupG was identified as a disaccharide carrying a phosphorylation at the C6 hydroxyl group of the non-reducing terminal MurNAc. The action of MupG produces MurNAc 6P, which is converted into GlcNAc 6P by the etherase MurQ, as well as GlcNAc. The faith of the second product remains so far unknown. Thus, the reaction catalyzed by MupG represents an additional step in the MurQ pathway compared to *E. coli* and *B. subtilis*, which is necessary in *S. aureus* to recycle the turnover product MurNAc-GlcNAc. The enzyme MupG

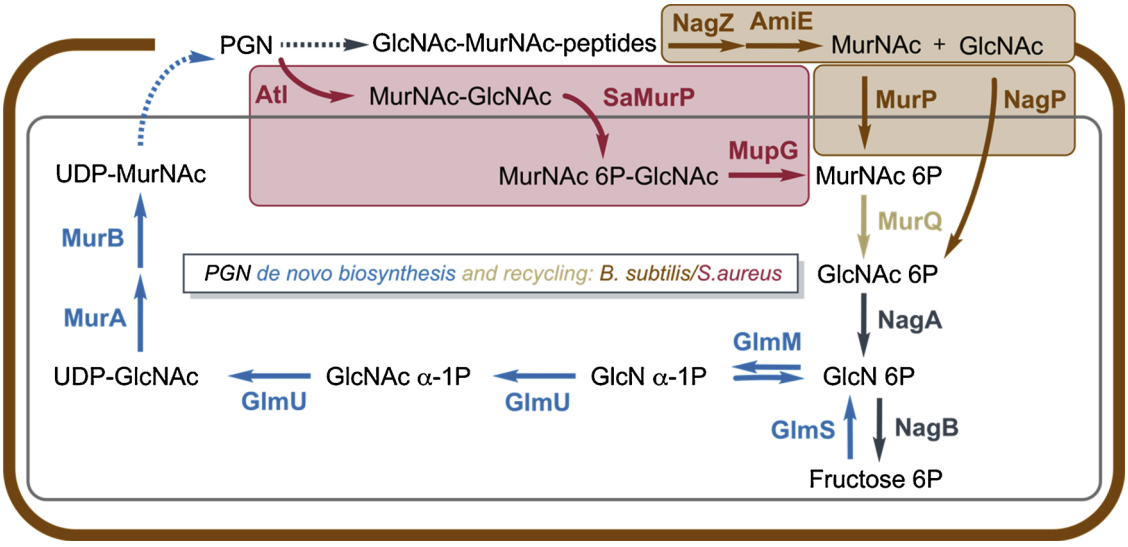

Fig. 7. Overview of the PGN recycling pathways of *B. subtilis* (brown) and *S. aureus* (red). Both Gram-positive bacteria recycle the MurNAc portion of their PGN via MurQ. In *B. subtilis*, the PGN is cleaved exhaustively outside the cell, yielding the sugars MurNAc and GlcNAc, which are taken up and phophorylated by PTS transporters MurP and NagP, respectively. In *S. aureus* a unique turnover product, the disaccharide MurNAc-GlcNAc is generated by the action of the major autolysin Atl, which is taken up and concomitantly phosphorylated by SaMurP and the product is cleaved in the cytoplasm by a novel MurNAc 6P hydrolase (MupG). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

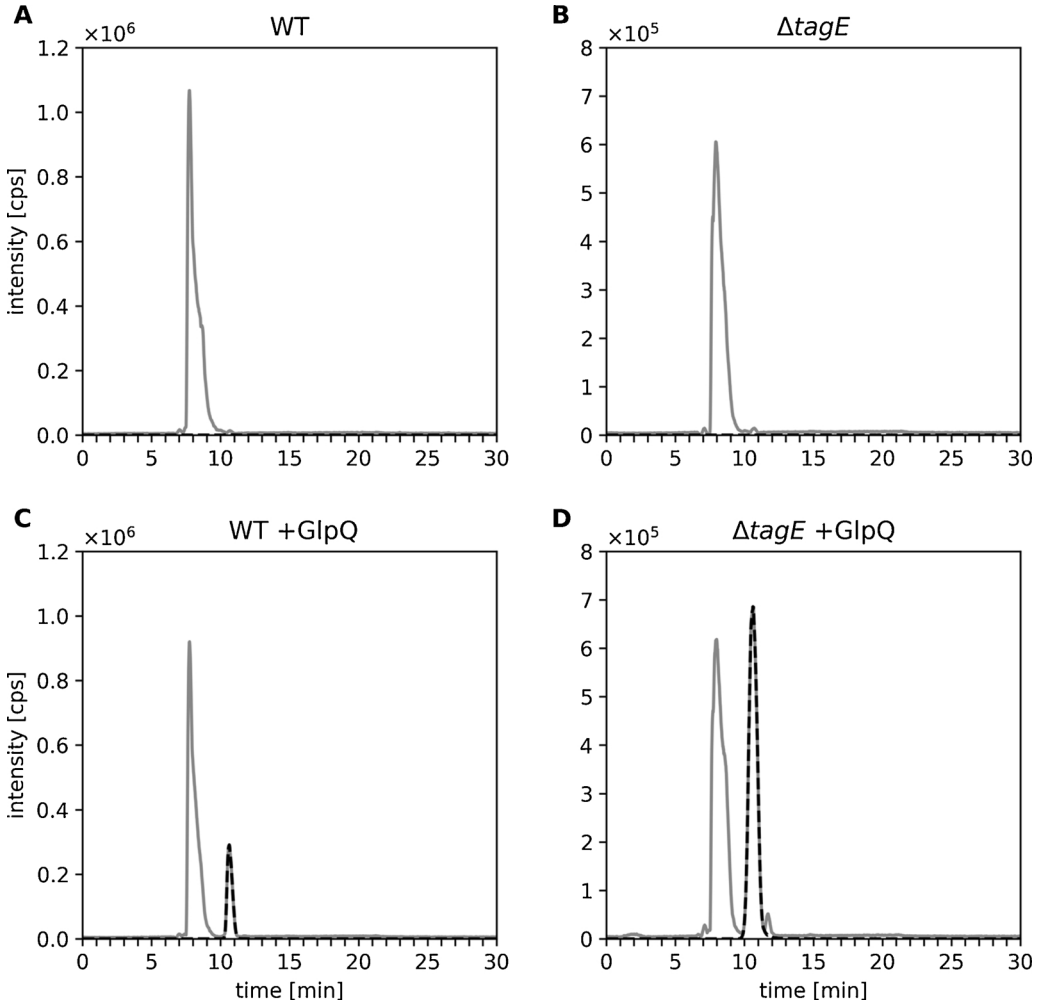

Fig. 8. GlpQ preferentially releases sn-glycerol 3P from WTA isolated from Δ*tagE* mutant cells over WTA from wild-type (WT) cells. Purified *B. subtilis* cell wall (100 µg, containing PGN and covalently bound WTAs) was incubated with GlpQ (1 µg) and the formation of reaction products was analyzed by HPLC-MS. Shown are the base peak chromatogramms (BPC; mass range $[M+H]^+$ = 100–1500 *m/z*; gray) and the extracted ion chromatogramms (EIC x 10^5 cps) of glycerol-phosphate (GroP) ($[M+H]^+$ = 173.022 +/- 0.02 *m/z*; blue). (A and C) wild type WTA (= partially glycosylated WTA) without GlpQ (control) and incubated with GlpQ incubated for 30 min. The peak area (area under the curve; AUC) of relased Gly-3P was AUC = 7.8 * 10^6. (B and D) non-glycosylated Δ*tagE* WTA without GlpQ (control) and with GlpQ incubated for 30 min. The obtained AUC = 29.8 × 10^6 was 3.8 times as much compared to the release of Gly-3P from WT WTA.

belongs to a so far unexplored protein family containing domain of unknown function 871 (DUF871) (Kluj et al., 2018) (Fig. 5). This protein family is narrowly distributed mostly among the Firmicutes, including Bacilli, but a MupG ortholog is absent in *B. subtilis*. Intriguingly, some bacteria contain multiple versions of MupG protein. The protein family divides in two distinct phylogenetic clades, one representing MupG and close homologs, and a second clade, with more distantly related orthologs (MupG-like proteins), may contain enzymes with different substrate specificities and functions (Kluj et al., 2018).

7. Degradation of wall teichoic acids and the role of the teichoicase GlpQ

Since recycling of the PGN in Gram-positive bacteria has been demonstrated, we questioned whether the wall teichoic acids (WTAs), which are covalently linked to the PGN, are also recovered. WTAs and PGN make up roughly equal amounts in the cell wall of Gram-positive bacteria. It has been shown that phosphate deficiency can be compensated by scavenging the phosphate stored within the WTA chain polymers (Grant, 1979). The reduction in WTA is accompanied by synthesis of phosphate-free teichuronic acids (TUA), consisting of poly-*N*-acetylglucosamine-glucuronic acid. About 70% of WTA are exchanged with TUA during phosphate starvation (Bhavsar et al., 2004; Ellwood and Tempest, 1969; Lang et al., 1982; Soldo et al., 1999). This WTA-to-TUA-shift is mediated by the two-component system PhoPR. The sensor kinase PhoR is inhibited by WTA biosynthesis intermediates, which are abundant during phosphate excess. When phosphate gets limiting, these intermediates are reduced and PhoR is able to phosphorylate the response regulator PhoP (Devine, 2018). The phosphorylated response regulator PhoR subsequently inhibits the transcription of WTA biosynthesis genes and activates transcription of TUA biosynthesis genes (Botella et al., 2014, 2011; Götz et al., 2014). Although it is long known that WTAs are degraded during phosphate starvation, the process is so far not completely understood. Proteome studies with *B. subtilis* have shed some light on that question. Cells were grown under phosphate limitation supplemented with purified WTA fragments and the proteome was compared with cells grown in phosphate excess (Unsleber, Franz-Wachtel, Macek, Mayer, unpublished results). Amongst other proteins, e.g. the alkaline phosphatase PhoB, the phosphodiesterases GlpQ and PhoD, were found to be highly upregulated (Unsleber, 2017). GlpQ and PhoD are controlled by the PhoPR regulon and preferentially degrade non-glycosylated WTAs (Myers et al., 2016; Unsleber, 2017). While GlpQ cleaves in an exo-acting manner releasing sn-glycerol 3-phosphate (Fig. 8), PhoD apparently catalyzes endo-hydrolysis at non-specific sites throughout the polymer (Myers et al., 2016). It has also been demonstrated that Δ*glpQ* or Δ*phoD* mutants retained WTA and ceased growth upon phosphate limitation, suggesting that they are key enzymes in WTA degradation (Myers et al., 2016). Interestingly, GlpQ is not able to use LTA as a substrate. The crystal structure of GlpQ reveals a binding cleft that specifically accommodates sn-glycerol 3-phosphate, which explains the stereochemical differentiation between WTA and LTA (Unsleber, 2017) (Walter & Mayer, unpublished). However, GlpQ is not only involved in WTA degradation. In *S. aureus* GlpQ permits growth within the host, where nutrient levels like glucose fluctuate. GlpQ is responsible for extracellular phosphodiesterase activity, by which *S. aureus* can scavenge glycero-phosphodiesters (GPD)

within body fluids. The released glycerol-3-phosphate serves as a carbon and phosphate source (Jorge et al., 2017, 2018). GlpQ is also involved in the general glycerol metabolism as part of the *glp* regulon, induced by glycerol-phosphate (Nilsson et al., 1994).

8. Conclusion

All bacteria - Gram-negative bacteria and Gram-positives - degrade their own cell wall by specific sets of autolytic enzymes, which is a prerequisite for bacterial growth and differentiation. As different as the sets of autolytic enzymes, are the released turnover fragments: diverse disaccharides, disaccharide-peptides (anhydromuropeptides, GlcNAc-MurNAc-peptides and MurNAc-GlcNAc-peptides), or after further extracellular cleavage, monosaccharides and peptides/amino acids. Bacteria generally are also able to recycle their cell wall turnover products using different strain-specific routes. They use specific sets of transporters, which may or may not phosphorylate the incoming fragments. Intracellularly, the fragments may be further degraded (catabolic recycling; MurQ) or channeled into the biosynthesis pathways (anabolic recycling; AmgK/MurU). This distinguishes Gram-negative bacteria, including the model organisms *E. coli* and *Pseudomonas* sp., whereas *T. forsythia* harbors both pathways. Gram-positive bacteria (*B. subtilis* and *S. aureus*) lack the anabolic recycling route but mostly contain a MurQ etherase. *B. subtilis* is distinguished by the ability to cleave the cell wall to monosaccharides and use specific transporters for these, whereas many other firmicutes bacteria, including *S. aureus*, apply the novel phospho-muramidase MupG enzymes to intracellularly degrade phosphorylated MurNAc-GlcNAc. Thus, although the ability to recycle their own cell wall is a general feature of bacteria, different recovery and reutilization strategies are applied.

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