Bioorganic Chemistry 54 (2014) 44–50

Contents lists available at ScienceDirect

Bioorganic Chemistry

journal homepage: www.elsevier.com/locate/bioorg

Minireview

Chemical biology of peptidoglycan acetylation and deacetylation

Patrick J. Moynihan [1], David Sychantha, Anthony J. Clarke *

Department of Molecular & Cellular Biology, University of Guelph, Guelph, Ontario N1G 2W1, Canada

ARTICLE INFO

Article history:
Received 12 March 2014
Available online 8 April 2014

Keywords:
Peptidoglycan
Murein
O-acetylation
N-deacetylation
O-acetyltransferase
O-acetylesterase
New antibiotic targets

ABSTRACT

Post-synthetic modification of the bacterial cell wall represents an important strategy for pathogenic bacteria to evade innate immunity and control autolysins. Modifications to the glycan backbone of peptidoglycan are generally restricted to the C-6 hydroxyl and C-3 amino moieties, with the most common being acetylation and deacetylation. In this review we discuss the pathways for O-acetylation, de-O-acetylation and N-deacetylation with an emphasis on the chemical–biological approaches used in their investigation. The current challenges in the field and the prospects of targeting these systems with novel therapeutics are also explored.

Contents

1. Introduction

The cell envelope defines a bacterium and protects it from both its own internal turgor pressure and external threats. These threats can include chemicals (such as antibiotics), host defense agents (such as lysozyme and complement), or direct attacks through type VI secretion systems and bacteriophage, amongst others. One of the most important components of the cell envelope is the essential peptidoglycan (PG) sacculus.

The PG sacculus is a single macromolecule that encompasses the entire bacterial cell wall. As its name implies, PG is heteropolymeric being composed of glycan chains that are covalently linked together through short peptides (Fig. 1). The backbone of the glycan strands is a repeating unit of 2-acetamido-2-deoxy-D-glucopyranose (*N*-acetylglucosamine; GlcNAc) linked β-(1 → 4) to 2-acetamido-3-*O*-[(1*R*)-1-carboxyethyl]-2-deoxy-D-glucopyranose (*N*-acetylmuramic acid; MurNAc) that terminate at their reducing ends, at least in Gram negative bacteria, with 1,6-anhydro-2-acetamido-3-*O*-[(1*R*)-1-carboxyethyl]-2-deoxy-D-glucopyranose (1,6-anhydro-*N*-acetyl-muramic acid; AnhMurNAc) residues. When first prepared biosynthetically, a "stem" pentapeptide of

Abbreviations: PG, Peptidoglycan; GlcNAc, 2-acetamido-2-deoxy-d-glucopyranose (*N*-acetylglucosamine; MurNAc, 2-acetamido-3-*O*-[(1*R*)-1-carboxyethyl]-2-deoxy-d-glucopyranose; AnhMurNAc, 1,6-anhydro-2-acetamido-3-*O*-[(1*R*)-1-carboxyethyl]-2-deoxy-d-glucopyranose (1,6-anhydro -*N*-acetyl-muramic acid; LT, lytic transglycosylase; Oat, *O*-acetyltransferase; Pat, peptidoglycan *O*-acetyltransferase; Ape, *O*-acetylpeptidoglycan esterase; *p*NP-Ac, *p*-nitrophenyl-acetate; 4MU-Ac, 4-methylumbelliferyl-acetate; PBP, penicillin-binding protein.

* Corresponding author. Fax: +1 (519) 837 1802.
E-mail address: a.clarke@exec.uoguelph.ca (A.J. Clarke).
[1] Present address: School of Biosciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK.

http://dx.doi.org/10.1016/j.bioorg.2014.03.010
0045-2068/

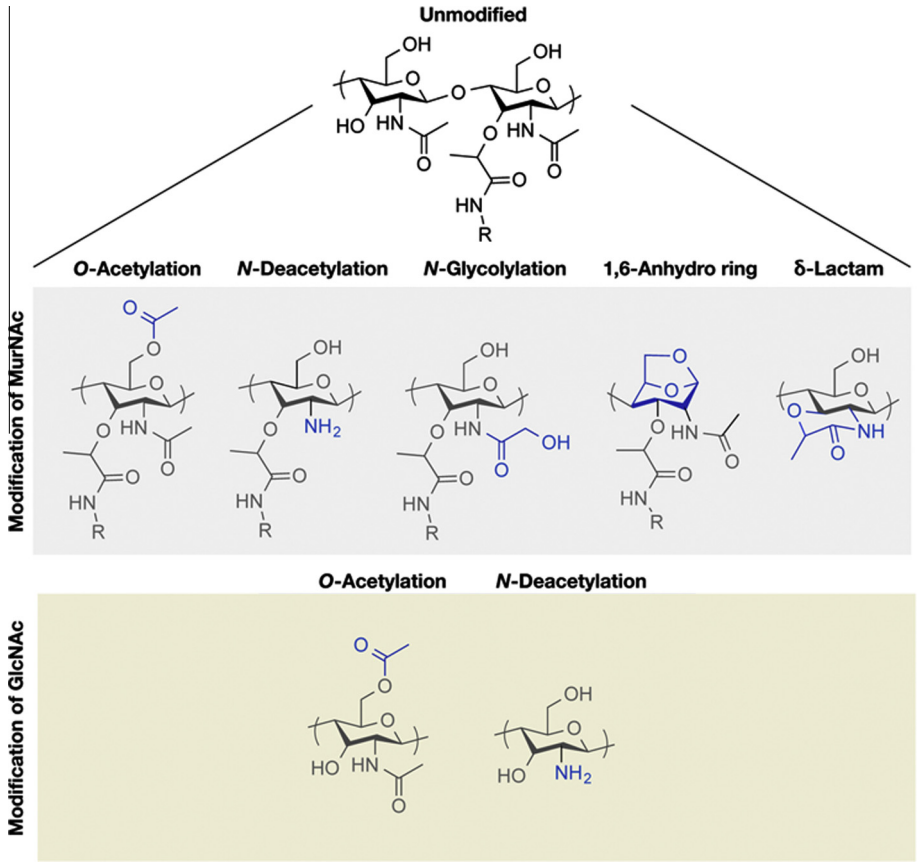

Fig. 1. Modifications of the PG glycan strand. Post-synthetic modifications of the glycan chain are found in both Gram-positive and Gram-negative bacteria. Most of these structural variations provide cells with the ability to regulate or inhibit endogenous and exogenous lytic enzymes, respectively, but the formation of 1,6 anhydro- and δ-lactam MurNAc are the result of metabolic activities. Modifications are colored in blue.

alternating L- and D-amino acids is attached to the C-3 lactyl moiety of MurNAc, which ends at its C-terminus with D-Ala-D-Ala. Crosslinks between the stem peptides covalently link neighboring glycan strands together thereby forming the single macromolecular sacculus. The composition of the stem peptide and the nature of crosslinking is generally well conserved amongst closely related bacteria. Although variation can occur within an individual organism, stem peptide composition and crosslinking typically diverge along phylogenetic lines [1]. These variations observed in stem peptide composition and crosslinking form the basis of a naming convention for the different PGs found in nature [1].

The description provided above presents the general "textbook" definition of PG but, despite the general consistency of its repeating units, PG composition is very complex, even within a single strain of a bacterium. For example, the lysozyme digestion of PG from a single *Escherichia coli* culture results in the generation of more than 70 distinct muropeptide species that can be resolved by reverse-phase HPLC [2]. This complexity is further compounded by the variability of PG composition that occurs within bacteria with changes to environmental factors, growth condition and/or age, and that such modifications are non-stoichiometric. Some of these changes can have significant consequences to the clinician attempting to treat a bacterial infection. One of the most dramatic examples of such would be with vancomycin resistant enterococci (VRE) where the simple substitution of the terminal D-Ala residue of stem pentapeptides with D-lactate precludes effective binding of vancomycin [3]. A further complicating factor associated with PG structure and composition concerns its metabolism. Often viewed as an inert mesh-like bag, PG is in fact a highly dynamic macromolecule. For example, cell wall turnover rates as high as 50% have been observed in cultures of *Neisseria gonorrhoeae* [4] where older material is released from the sacculus as new precursor components are ligated in. The timing and localization of the host of enzymes and proteins involved in these processes of lysis and biosynthesis have to be exquisitely coordinated and controlled so that cell rupture is avoided. As this occurs, much of the released PG metabolites are recycled back into the cell involving a separate coordinated group of enzymes and proteins. This balance of catabolic and anabolic events has been shown to be essential for numerous processes including the insertion of flagella, pili and secretory systems and for transitioning into swarmer cells [5–7], in addition to the general growth and ultimate division of bacterial cells. A number of reviews have been published recently on the structure and architecture of peptidoglycan [8–10], its biosynthesis [11,12] and the lytic enzymes involved [13,14] and the reader is directed to them to gain a more complete understanding of the diversity of this essential wall component and the complexity of its metabolism. This review, however, focuses on post-synthetic modifications that occur to the glycan backbone of the PG sacculus and how the enzymes associated with these events may serve as novel targets for antibacterial therapy.

2. Post-synthetic modifications: General

Post-synthetic modifications to the PG glycan backbone are generally limited to the C-2 amine or the C-6 hydroxyl moieties of either aminosugar. For example, *N*-deacetylation of GlcNAc is observed in many species of bacteria, particularly amongst Gram-positive species, while *N*-glycolylation of GlcNAc is a

hallmark of *Mycobacterium tuberculosis* and other closely related organisms [15,16] (Fig. 1). Attachment of teichoic acids, other surface polymers such as capsular polysaccharides and arabinogalactans and O-acetylation have all been observed at the C-6 hydroxyl moiety of MurNAc residues [17–19]. Without exception, these and other modifications to the PG glycan protect it from lysis by both exogenous host-defense agents, such as the lysozymes of host innate immune systems, and the endogenous autolysins involved in PG metabolism [20,21]. Indeed, the absolute requirement of an intact PG sacculus has been viewed as the "bacterial Achilles heel" [22], and organisms that wish to kill bacteria have taken advantage of this. With the lysozymes (EC 3.2.1.17; peptidoglycan *N*-acetylmuramoylhydrolase), the coordination and binding of PG into its active site cleft is directed by H-bonding between amino acid residues that line the six binding subsites of the enzyme and both the *N*-acetyl and the C-6 hydroxyl groups of three successive GlcNAc and MurNAc residues, respectively [23]. The N-deacetylation of the GlcNAc residues thus precludes the opportunity for this productive binding while the acylation of the C-6 hydroxyl sterically blocks the appropriate alignment of the glycosidic linkage to be cleaved at the reactive center of the enzyme. With both, a direct relationship has been demonstrated between the increasing level of modification and increasing resistance to lysozyme-induced lysis [reviewed in 24]. Thus, in the extreme cases such as that with pathogenic strains of *Staphylococcus aureus* where O-acetylation levels exceed 70% (of total Mur content), cells are virtually resistant to lysozyme hydrolysis [25].

The autolysins, on the other hand, are the collection of bacterial lytic enzymes involved in PG metabolism and they are so named because their uncontrolled activity results in lysis of the bacterium. It is this eventuality that is exploited by some antibiotic therapies, particularly with the β-lactams, where the delicate balance between synthesis and degradation is affected to achieve bacterial lysis. Thus, as the crosslinking of PG is halted by β-lactams, the autolysins (many of which are not inhibited by this class of antimicrobials) continue to cleave the PG resulting in a weakened sacculus that eventually ruptures due to the cellular turgor pressure [26]. The autolysins that are active against the glycan backbone of PG include the 1,4-β-*N*-acetylglucosaminidases (EC 3.2.1.14; (1 → 4)-2-acetamido-2-deoxy-β-D-glucan glycanohydrolase) and the lytic transglycosylases (EC 4.2.2.x; LT), which are predominant in Gram-positive and Gram-negative bacteria, respectively. The LTs have specificity for the same glycosidic linkage as the lysozymes, *viz.* that between MurNAc and GlcNAc residues, and they share the "lysozyme" structural fold [27]. Intriguingly however, they are not hydrolases, but rather they cleave the β-(1 → 4) linkage with the concomitant formation of 1,6-anhydromuramoyl residues (Fig. 1) thus involving different catalytic mechanisms. Clearly, the presence of any modification at the C-6 hydroxyl group on MurNAc residues would preclude LT activity, and consequently we have postulated that O-acetylation provides control of LT activity at the substrate level [21]. Whereas it has yet to be demonstrated experimentally, we also propose that the N-deacetylation of GlcNAc residues that occurs predominantly in Gram-positive bacteria would inhibit the activity the 1,4-β-*N*-acetylglucosaminidases, the major autolysin of these bacterial cell types.

3. N-deacetylation

N-deacetylated PG is found mostly in Gram-positive bacteria (Fig. 2). It can occur at either MurNAc or GlcNAc and individual bacteria have been identified with both modifications [28]. To date, the only Gram-negative bacterium known to contain N-deacetylated PG is *Helicobacter pylori* [29]. The degree of N-deacetylation varies between <20% and 80% relative to GlcN, and <20% and 50% relative to MurN, in different species of *Bacillus* [30].

The N-deacetylation of PG is catalyzed by *N*-acetyl-D-glucosamine amidohydrolase (EC 3.5.1.33; *N*-acetylglucosamine deacetylase) as first demonstrated by Roseman almost 60 years ago [31]. These enzymes appear to have a relaxed substrate specificity which is best exemplified by PgdA from *Streptococcus pneumoniae* [32]. This enzyme was observed to N-deacetylate PG, chitooligosaccharides, *p*-nitrophenol acetate (*p*NP-Ac) and 4-methylumbelliferyl acetate (4MU-Ac). Such broad specificity likely accounts for the inclusion of these N-deacetylases in Carbohydrate Esterase family 4 (CE4) of the CaZY classification system and database (www.cazy.org).

The biological role of N-deacetylated PG has, until recently been implicated only in lysozyme resistance. Recent studies, however, with *Bacillus anthracis* and *Bacillus cereus* revealed that this post-synthetic modification might have physiological function(s). Of the ten hypothetical polysaccharide deacetylase homologs identified in these bacilli, five were investigated using both genetic (knockout) and biochemical approaches. Three were confirmed to function as authentic PG (GlcNAc) *N*-deacetylases, and two of these were observed to influence cell shape and cell separation, but surprisingly neither appeared to contribute to lysozyme resistance [32,33].

The impact of N-deacetylation of MurNAc is still uncertain and it is doubtful that it would have any role in conferring resistance to lysozyme. Whereas lysozyme is known to require the N-acetyl moiety of GlcNAc to bind its substrate [23], the same moiety on MurNAc would not be available for similar interactions based on the known crystal structure of the enzyme in complex with ligands and the observed orientation of PG ligands in its active site cleft [23,34]. Hence, it is possible that the deacetylation of MurNAc residues may also play a physiological role in PG metabolism but clearly more research is required to establish this. The impetus for such studies may be the recent finding that a virtual high-throughput screen led to the identification of several inhibitors of the *S. pneumoniae* PgdA that were assessed biochemically and found to have IC_{50} values towards this pathogen in the micromolar range [35].

4. O-acetylation

The O-acetylation of PG occurs at the C-6 hydroxyl of MurNAc residues in most organisms with modification of GlcNAc observed in some species of *Lactobacillus* and possibly *Bacillus* [36,37]. In organisms that O-acetylate their PG, the modification is typically present on 20–70% of MurNAc residues depending on the species, strain, and growth conditions. Whereas all Gram-positive and most Gram-negative bacteria do O-acetylate their PG, *E. coli* and *Pseudomonas aeruginosa* and closely related Gram-negative species are amongst the few exceptions that do not [38]. It is likely that in place of substrate modification, these organisms utilize proteinaceous inhibitors that are localized to the periplasm to control their lytic enzymes ([38,39]). Furthermore, it is not yet clear if O-acetylation of PG is dispersive throughout the sacculus or localized to particular cellular regions, such as the poles. It is abundantly clear, however, that O-acetylation is important for pathogenesis. Work by Bera et al. [40] demonstrated that the ability to O-acetylate PG is correlated with pathogenicity and that the modification is a substantial determinant of lysozyme resistance. O-Acetylation also has been shown to be an important virulence factor in *Listeria monocytogenes* [41]. In Gram-negative bacteria, the presence of O-acetylation has been implicated in PG chain-length regulation, as well as the control of autolysins [42,43].

Until 2005, very little was known about the pathway(s) for PG O-acetylation. Early biochemical evidence indicated that it is a maturation event occurring after the assembly (*viz.* transglycosylation and transpeptidation) of PG precursors into the existing

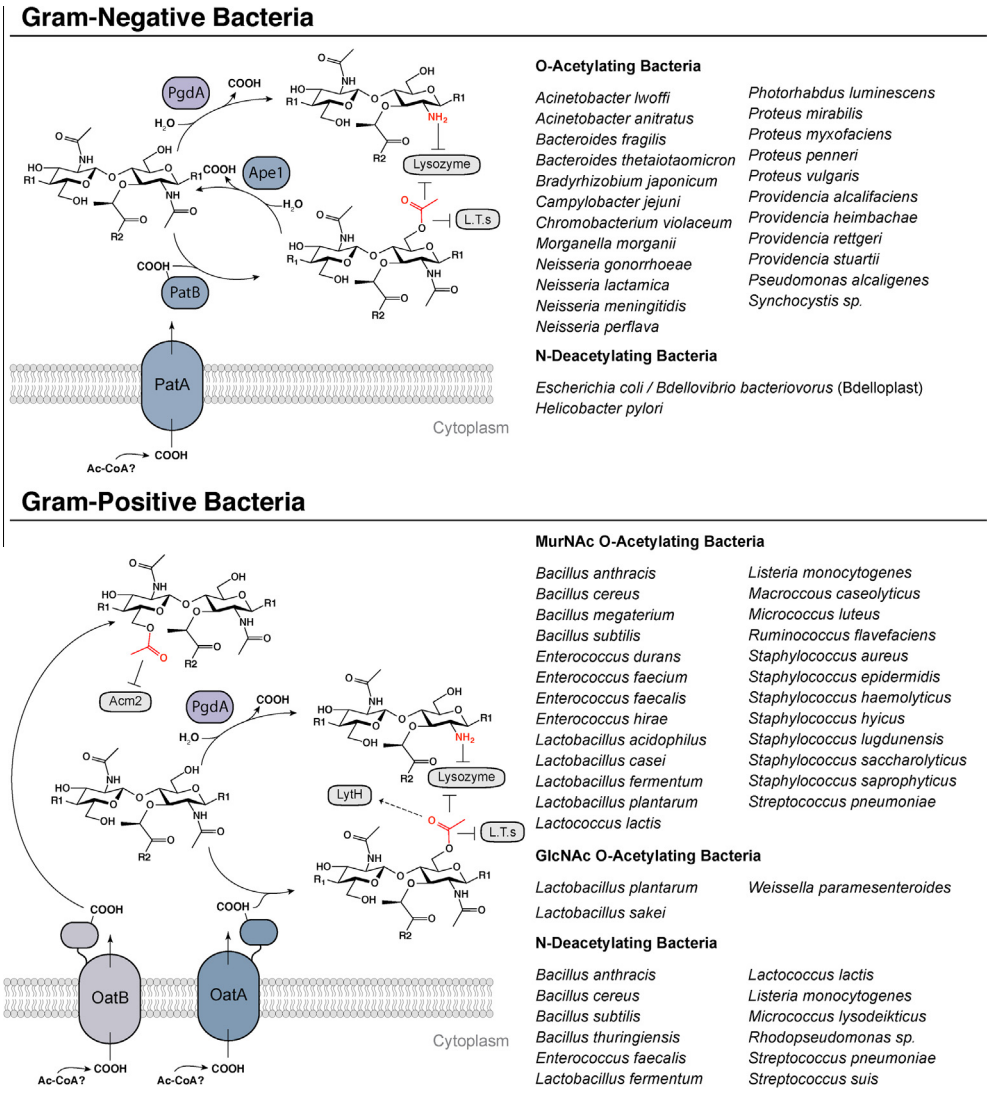

Fig. 2. PG acetylation and deacetylation. The O-acetylation of PG is catalyzed by different enzymatic systems in Gram-negative and Gram-positive bacteria. In Gram-negative bacteria, acetate is transferred to the periplasm by PatA for use by PatB to modify PG. Ape1 removes the acetate as required for LT activity by the organism. In Gram-positive bacteria, O-acetylation is catalyzed by a single bimodal protein, OatA. The N-terminal domain of OatA is proposed to act in a manner similar to PatA while the C-terminal domain is predicted to function as the acetyltransferase. The O-acetylation of GlcNAc, thus far only confirmed with *Lactobacillus plantarum*, regulates the activity of that organism's major autolysin Acm2, which is an *N*-acetylglucosaminidase. This modification is performed by a homolog of OatA named OatB. The organisms listed to the right are those in which the modification has been experimentally demonstrated. Notable exceptions for both types of modifications are *E. coli* and *P. aeruginosa*. R_1 – Extended glycan chain or terminal sugar moiety; R_2 – peptide side chain.

sacculus [24]. Thus, it was recognized that the source of acetate for this modification must be transported from the cytoplasm to the PG sacculus through the cytoplasmic membrane. Progress was finally made with the release of the genome sequences of *S. aureus* and *N. gonorrhoeae* in 2005 that led to the identification of two distinct PG O-acetylation systems specific to Gram-positive and Gram-negative bacteria, respectively.

Bera et al. [25] identified the *oatA* gene as being responsible for O-acetylation of muramoyl residues in *S. aureus* (Fig. 2). Homologs of OatA have been identified in several other Gram-positive bacteria, including species of *Streptococcus* [44], *B. cereus* and *B. anthracis* [37], *Lactococcus lactis* [45] Veiga et al., 2007) and *Lactobacillus plantarum* [45] Bernard et al., 2011). The *oatA* gene encodes a hypothetical integral membrane protein that is predicted to contain two domains. The N-terminal half is classified as an acyltranslocase and it possesses 11 hypothetical transmembrane helices [25]. The C-terminal module appears to be globular and it is predicted to be surface exposed. A lysine rich region in this module has been proposed to be the site of catalytic activity [40] but closer analysis suggests that it has the fold of SGNH/GDSL hydrolases, complete with a signature catalytic triad of Asp, His and Ser residues. Preliminary data support the importance of the Asp and Ser in conferring O-acetylation [45] (unpublished data).

A paralog of OatA, named OatB, was identified in *B. anthracis* by Laaberki et al. [37] and it was demonstrated to contribute to the overall level of O-acetylation of PG, but no obvious phenotype was observed with a deletion mutant. It was speculated that OatB was modifying GlcNAc although this was not confirmed experimentally. Bernard et al. [36] identified a different enzyme, also named OatB, in *L. plantarum*, *Lactobacillus sakei* and *Weissella paramesenteroides*. Phenotypic analysis of mutant strains lacking the *oatA* and *oatB* genes showed that they were required for the O-acetylation of MurNAc and GlcNAc, respectively. Other experiments in the same report showed that while the O-acetylation of MurNAc contributed to lysozyme resistance in *L. plantarum*, O-acetylation of GlcNAc did not. Importantly, direct evidence for

the O-acetylation of PG by these enzymes is still lacking. No biochemical analysis of either enzyme has been reported; in fact, until recently no *in vitro* assay had been developed to monitor the O-acetylation of any extracellular polysaccharide. Nonetheless, the mode of action of OatA and OatB is postulated to involve the transfer of acetate from cytoplasmic pools of acetyl-CoA to acceptor sites on PG following their translocation across the cytoplasmic membrane (Fig. 2).

Gram-negative bacteria do not encode homologs of OatA or OatB but instead they produce a second family of enzymes for PG O-acetylation. Using *N. gonorrhoeae* as the model system, Weadge et al. [46] first identified a cluster of genes, named *oap* for O-acetylation of PG, that encode hypothetical acetyltransferases. PG *O*-acetyltransferase (Pat) A was identified as a member of the MBOAT family but it appears to be composed of only a single module involving ten hypothetical membrane-spanning helices. Notably, it does not share significant sequence similarity with OatA. PatB, on the other hand, is a peripheral membrane protein localized to the periplasm. Shortly after this initial report, a second group identified the same genes as responsible for PG O-acetylation in *N. gonorrhoeae* but unfortunately they named them *pacA* and *pacB* (for peptidoglycan acetylase) [47]. We recommend the former nomenclature be adopted given the convention already established for other acetyltransferases, including the Oat enzymes. In addition to being present in Gram-negative bacteria, *oap* clusters are present in some species of the Gram-positive genus *Bacillus*, including *B. anthracis* [37,46]. With *B. anthracis*, both Oat and Pat systems have been demonstrated to be required to achieve wild-type levels of O-acetylation of its PG [37].

PatA remains uncharacterized, but PatB from *N. gonorrhoeae* has been the subject of an initial biochemical study [48]. The key findings of those experiments included confirmation that the enzyme is localized to the periplasm, and that its activity *in vivo* appears to be dependent upon a functional acetate translocator, such as PatA. Thus PatA and PatB are postulated to function as a two-component system for PG O-acetylation (Fig. 2). As an integral membrane protein, PatA would function like OatA or OatB to translocate acetate across the cytoplasmic membrane for PatB in the periplasm and its subsequent transfer to PG. Confirmation of this pathway awaits a detailed study of the mechanism of PatA activity and a clear demonstration of an interaction between PatA and PatB.

Prior to our study of PatB, and despite knowing of the PG O-acetylation for over 50 years [17,49], no *in vitro* biochemical characterization of any extracellular *O*-acetyltransferase had been performed because no *in vitro* assay for acetyltransferase activity had been developed. This situation reflected the challenges with reconstituting integral membrane proteins that functions with a sparingly soluble protein to transfer acetate to an insoluble substrate such as PG (PG is insoluble in all solvents including DMSO or boiling SDS solution). However, a break though was achieved recently when it was found that PatA could be replaced with chromogenic pseudosubstrate acetate donors, such as *p*NP-Ac or 4MU-Ac, and that PatB is capable of transferring this acetate to chitooligosaccharide acceptors *in vitro* [50]. The development of this assay has provided the means for a detailed biochemical analysis of both PatB (manuscript submitted) and OatA (manuscript in preparation), including important insights into their substrate specificities. Using this chemical-biological approach, our preliminary data indicate that PatB uses a catalytic triad of Ser-His-Asp in its mechanism of action to transfer acetate to chitooligosaccharides in a manner that is reverse to the reaction catalyzed by *O*-acetylpeptidoglycan esterases [51,52], as described below.

Little is known about the regulation of PG O-acetylation at either the genetic or enzyme level. *In silico* analysis of the *oap* cluster in *N. gonorrhoeae* suggests that the genes are transcribed from a common promoter [46], a hypothesis that was later confirmed by Veryier et al. [42], but the nature or existence of an inducer/regulator remains unknown. With the Gram-positives, the expression of *oatA* in *L. lactis* has been linked to cell-wall stress [45]. A two-component system, CesSR, is responsible for sensing cell-wall damage. This system up-regulates the expression of *spxB* which leads to increased production of OatA. This finding clearly illustrates the importance of PG O-acetylation in terms of bacterial response to host immunity.

Another level of control for PG O-acetylation, at least for Gram-negative bacteria, may be the association of the acetyltransferases with PG "replicases," membrane-bound complexes of enzymes responsible for PG biosynthesis [53]. As evidence for this, PG O-acetylation was shown to decrease when penicillin-binding protein (PBP) 2, a transpeptidase required for PG maturation and a component of the PG replicases, is inactivated [54]. This suggests an interaction between *O*-acetyltransferase proteins and the PBP. Clearly though, the regulation of expression and localization of the enzymes involved in PG O-acetylation require further detailed investigation.

5. O-deacetylation

In addition to encoding the PG *O*-acetyltransferases, the *oap* clusters of Gram-negative bacteria also encode *O*-acetylpeptidoglycan esterases (Ape) for the removal of the modification [46]. With a single-layered PG sacculus, the ability to remove localized O-acetylation by these bacteria would be essential for their continued growth and division which involves the generation of insertion sites by the lytic transglycosylases of the PG replicases for the incorporation of PG precursors. Presumably, this is not a requirement for the multi-layered PG sacculus of Gram-positive bacteria and moreover, being exposed to the environment, it is likely that the retention of the O-acetylation on their PG helps protect them from exogenous lysozymes.

Ape has recently been implicated in PG chain-length regulation where it was shown that *Neisseria meningitidis* cells increased in size when their *ape* gene had been knocked out [42]. Furthermore, *in vivo* studies showed that the enzyme has specificity for GlcNAc-MurNAc-tripeptide, although this specificity appears to be imparted by the organism rather than by the enzyme itself as expression of the enzyme in *E. coli* did not result in a similar specificity [42].

Again using *N. gonorrhoeae* as the model, the *ape* gene product was confirmed to have specificity as an *O*-acetylPG esterase [51].

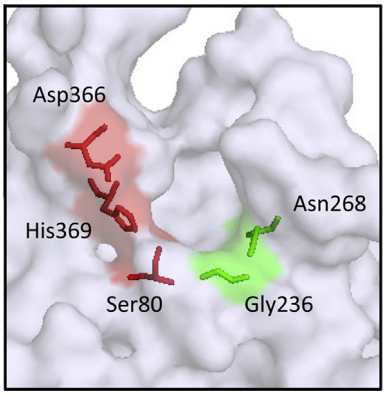

Fig. 3. Identification of catalytic residues in *N. gonorrhoeae* Ape1. The catalytic triad residues within the predicted three-dimensional structure of Asp, His, and Ser are depicted in red and those that constitute the oxyanion hole to stabilize the putative transition state are in green. The structure was predicted by Modeller.

The availability of soluble substrate analogs, such as *p*NP-Ac, together with the soluble nature of the enzyme again permitted a detailed characterization of their mechanism of action which indicates that they belong to the GDSL superfamily of serine esterases and thus use a catalytic triad of Ser, His and Asp residues to catalyze the release of acetate from PG. This mechanism of action was confirmed and the identification of the catalytic residues was made [55], including those that constitute the oxyanion hole that would stabilize the putative transition state [52] (Fig. 3). Hence, the Gram-negative bacteria would appear to balance the expression and activities of their Pat and Ape enzymes to provide sites for cleavage by the LTs as required. Evidence for this was reported recently when it was shown that *ape* gene is expressed together with the *pat* genes as part of the *oap* operon. How the activity of the Ape esterases is localized and controlled to permit site-specific cleavage of PG is currently unknown, but it is possible that they too comprise the PG replicases, at their leading front. Another finding of Veyrier et al. demonstrated that Ape is restricted *in vivo* to deacetylating GlcNAc-MurNAc-tripeptide, which may have implications in PG chain length regulation [42].

Given the apparent importance of Ape in the metabolism of O-acetylated PG, it would be expected that Ape is required for normal growth of Gram-negative bacteria with this modification, and that the enzyme may serve as new antibacterial target. To validate this proposal, a fluorogenic assay using 4MU-Ac as substrate was made amenable for high-throughput screening [56]. Using this assay, Ape was screened against a subset of compounds of the Canadian Compound Collection at the McMaster HTS Laboratory. After identifying a number of false-positives, several compounds were found to effectively inhibit the enzyme with apparent K_i values ranging from 65 nm to 7 μM. The identified inhibitors were an eclectic group of heterocyclic compounds with little commonality amongst them. However, one compound, the anthraquinone purpurin (1,2,4-trihydroxyanthraquinone; a natural dye isolated from roots of the madder plant (*Rubia tinctorum*)) was selected for further study because no other anthraquinone in the screen appeared to serve as a potent inhibitor. This list included alizarin (1,2-dihydroxyanthraquinone) which simply lacks the 4-hydroxy group of purpurin (Fig. 4). Nonetheless, the inhibitory constants of Ape for the three were different by greater than an order of magnitude; compare 1.7 μM and 50 μM at pH 6 for purpurin and alizarin, respectively (unpublished data). The reason for this difference is not currently known but it may be related to the differences in the p*K* values of the 2-hydroxyl groups. Whereas there is no biologically relevant difference between the two 1-hydroxyl groups of the two anthraquinones, the p*K* values of the 2-hydroxyl are reported to be 4.7 and 6.6 for purpurin and alizarin, respectively [57]. Understanding this may have important consequences, because purpurin was found to alter cellular morphology and then inhibit the growth of only those bacteria, both Gram-positive and Gram-negative, that de-O-acetylate their PG [56].

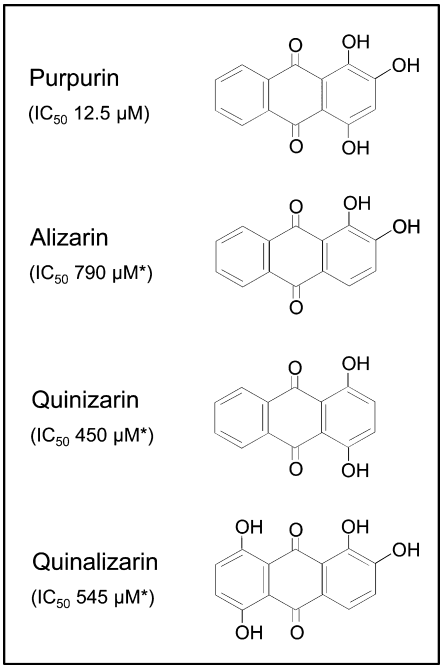

Fig. 4. Anthraquinone-based inhibitors of *N. gonorrhoeae* Ape1. The IC_{50} values were determined using 2 mM 4-nitrophenylacetate as substrate at pH 6.5. Due to solubility limitations, the values denoted by the asterisks had to be estimated from extrapolations of inhibition plots (data from [56] and unpublished).

6. Future directions

The most pressing need in the area of PG enzymology is that of defined, biologically relevant substrates. The complete exploration of the substrate specificity of a given enzyme requires a library of PG-related compounds ranging from uncross-linked material to cross-linked PG of varying sizes and linkages with similar variations in glycan chain-length. The heterogeneity of the PG sacculus in both an individual cell and a population of cells make purification of these molecules in practical quantities from bacteria extremely challenging. While separation *via* RP-HPLC can yield purified PG fragments, resolution of large molecules is poor, especially if the samples are not reduced. Significant headway has been made in the chemical synthesis of PG fragments (e.g., [58,59]), though the process is extremely challenging. For this reason there has been an increase in efforts to enzymatically synthesize PG *in vitro*. *In vitro* transglycosylation of Lipid II (the membrane bound PG precursor) has been achieved using enzymes from *S. pneumoniae*, *S. aureus* and *E. coli* [60–64]. The transpeptidation of this material *in vitro* has only been reported for *E. coli* and *S. pneumoniae* [65–69]. These studies have provided the basis for the ongoing search for a facile method for the generation of substrate for PG active enzymes.

The availability of homogeneous preparations of a true homopolymer of polymerized Lipid II will no doubt greatly facilitate investigations on the acetylation and deacetylation of PG. Indeed, recent studies in this aspect of PG research have been dramatically enhanced by the inclusion of chemical-biological agents. By taking advantage of substrate mimics the first-ever kinetic parameters for PG acetylases and deacetylases have been generated. Future studies should continue to exploit these opportunities as well as be focused on determining the crystal structure of the Pat, Ape and Oat-type enzymes; no structure is currently known for any of them despite the concerted efforts of a number of research groups. The issue appears to be one of mosaicity, and neither the chemical modification of surface residues, amino acid replacements through protein engineering techniques, nor co-crystallization with ligands has enhanced the quality of the protein crystals obtained. In the meantime, continuing studies into the structure and function relationship will provide information essential for the validation of these enzymes as new antibacterial targets, and then the identification of mechanism-based inhibitors that may serve as lead compounds to new classes of antibiotics. In this regard, we propose that the enzymes associated with the O-acetylation and deacetylation of PG represent excellent targets to pursue because: (i) their PG substrate is unique to, but common amongst, almost all bacteria; (ii) the MurNAc-GlcNAc substrate residues remain consistent in all PGs; (iii) the lack of variation in the glycan chain decreases opportunity for resistance to evolve, at least involving substrate

modification such as seen with VRE; (iv) they are required to control PG metabolism and, in the case of Gram-positive bacteria, provide protection from the lysozyme activity of innate immune systems; and (v) a mechanism-based inhibitor for any one of the enzymes will likely prove to be reactive against all in the same way that β-lactams bind all PG transpeptidases, albeit with varying affinities. Furthermore, even if inhibitors of these enzymes do not function effectively *in vitro*, they may provide an opportunity to work in synergy with the innate immunity systems by weakening PG and making it vulnerable to host attack. An added value is that targeting the acetylation/deacetylation systems focuses the antibacterial therapy largely on pathogenic organisms while not affecting much of the commensal flora.

Acknowledgments

Studies on the *O*-acetylation and de-*O*-acetylation of peptidoglycan in our laboratory are funded by a Team Grant to AJC from the Canadian Institutes of Health Research (TGC 114045), and an Ontario Graduate Scholarship to PJM from the Government of Ontario.

References

[1] K.H. Schleifer, O. Kandler, Bacteriol. Rev. 36 (1972) 407–477.
[2] B. Glauner, Anal. Biochem. 172 (1988) 451–464.
[3] T.D. Bugg, G.D. Wright, S. Durka-Malen, M. Arthur, P. Courvalin, C.T. Walsh, Biochemistry 30 (1991) 10408–10415.
[4] B.H. Hebeler, F.E. Young, J. Bacteriol. 126 (1976) 1180–1185.
[5] T. Nambu, T. Minamino, R.M. Macnab, K. Kutsukake, J. Bacteriol. 181 (1999) 1555–1561.
[6] H. Strating, C. Vandenende, A.J. Clarke, Can. J. Microbiol. 58 (2012) 1183–1194.
[7] H. Wehbi, E. Portillo, H. Harvey, A.E. Shimkoff, E.M. Scheurwater, P.L. Howell, et al., J. Bacteriol. 193 (2010) 540–550.
[8] R.D. Turner, W. Vollmer, S.J. Foster, Mol. Microbiol. 91 (2014) 862–874.
[9] W. Vollmer, S.J. Seligman, Trends Microbiol. 18 (2010) 59–66.
[10] W. Vollmer, D. Blanot, M.A. de Pedro, FEMS Microbiol. Rev. 32 (2008) 149–167.
[11] M.G. Pinho, M. Kjos, J.W. Veening, Nat. Rev. Microbiol. 11 (2013) 601–614.
[12] A.J. Egan, W. Vollmer, Ann. NY Acad. Sci. 1277 (2013) 8–28.
[13] W. Vollmer, B. Joris, P. Charlier, S. Foster, FEMS Microbiol. Rev. 32 (2008) 259–286.
[14] J.W. Johnson, J.F. Fisher, S. Mobashery, Ann. NY Acad. Sci. 1277 (2013) 54–75.
[15] I. Azuma, D.W. Thomas, A. Adam, J.M. Ghuysen, R. Bonaly, J.F. Petit, et al., Biochim. Biophys. Acta 208 (1970) 444–451.
[16] H. Hayashi, Y. Araki, J. Bacteriol. 113 (1973) 592–598.
[17] A. Abrams, J. Biol. Chem. 230 (1958) 949–959.
[18] L. Deng, D.L. Kasper, T.P. Krick, M.R. Wessels, J. Biol. Chem. 275 (2000) 7497–7504.
[19] J.L. Strominger, J.M. Ghuysen, Biochem. Biophys. Res. Commun. 12 (1963) 418–424.
[20] E.M. Scheurwater, C.W. Reid, A.J. Clarke, Int. J. Biochem. Cell Biol. 40 (2008) 586–591.
[21] P.J. Moynihan, A.J. Clarke, Int. J. Biochem. Cell Biol. 43 (2011) 1655–1659.
[22] J. Coyette, A. van der Ende, FEMS Microbiol. Rev. 32 (2008) 147–148.
[23] C. Blake, D. Koenig, G.A. Mair, A.C. North, D.C. Phillips, V.R. Sarma, Nature 206 (1965) 757–761.
[24] A.J. Clarke, C. Dupont, Can. J. Microbiol. 38 (1992) 85–91.
[25] A. Bera, S. Herbert, A. Jakob, W. Vollmer, F. Götz, Mol. Microbiol. 55 (2005) 778–787.
[26] Z. Yao, D. Kahne, R. Kishony, Mol. Cell 48 (2012) 705–712.
[27] E.J. van Asselt, A.J. Dijkstra, K.H. Kalk, B. Takacs, W. Keck, B.W. Dijkstra, Structure 7 (1999) 1167–1180.
[28] Y. Araki, T. Nakatani, H. Hayashi, E. Ito, Biophys. Res. Commun. 42 (1971) 691–697.
[29] G. Wang, A. Olczak, L.S. Forsberg, R.J. Maier, J. Biol. Chem. 284 (2009) 6790–6800.
[30] G.F. Zipperle, J.W. Ezzell, R.J. Doyle, Can. J. Microbiol. 30 (1984) 553–559.
[31] S. Roseman, J. Biol. Chem. 226 (1957) 115–124.
[32] S. Balomenou, A. Fouet, M. Tzanodaskalaki, Mol. Microbiol. 87 (2013) 867–883.
[33] E. Psylinakis, J. Biol. Chem. 280 (2005) 30856–30863.
[34] C.C. Blake, L.N. Johnson, G.A. Mair, A.C. North, D.C. Phillips, V.R. Sarma, Proc. R. Soc. Lond. B Biol. Sci. 167 (1967) 378–388.
[35] N.K. Bui, S. Turk, S. Buckenmaier, F. Stevenson-Jones, B. Zeuch, S. Gobec, W. Vollmer, Biochem. Pharmacol. 82 (2011) 43–52.
[36] E. Bernard, T. Rolain, P. Courtin, A. Guillot, P. Langella, P. Hols, M-P. Chapot-Chartier, J. Biol. Chem. 286 (2011) 23950–23958.
[37] M.-H. Laaberki, J. Pfeffer, A.J. Clarke, J. Dworkin, J. Biol. Chem. 286 (2011) 5278–5288.
[38] C.A. Clarke, E.M. Scheurwater, A.J. Clarke, J. Biol. Chem. 285 (2010) 14843–14847.
[39] L. Callewaert, A. Aertsen, D. Deckers, K.G.A. Vanoirbeek, L. Vanderkelen, J.M. Van Herreweghe, B. Masschalck, D. Nakimbugwe, J. Robben, C.W. Michiels, PLoS Pathog. 4 (2008) e1000019.
[40] A. Bera, R. Biswas, S. Herbert, F. Götz, Infect. Immun. 74 (2006) 4598–4604.
[41] C. Aubry, C. Goulard, M.-A. Nahori, N. Cayet, J. Decalf, M. Sachse, I.G. Boneca, P. Cossart, O. Dussurget, J. Infect. Dis. 204 (2011) 731–740.
[42] F.J. Veyrier, A.H. Williams, S. Mesnage, C. Schmitt, M.-K. Taha, I.G. Boneca, Mol. Microbiol. 87 (2013) 1100–1112.
[43] H. Strating, A.J. Clarke, Anal. Biochem. 291 (2001) 149–154.
[44] M.L. Crisóstomo, W. Vollmer, A.S. Kharat, S. Inhülsen, F. Gehre, S. Buckenmaier, A. Tomasz, Mol. Microbiol. 61 (2006) 1497–1509.
[45] P. Veiga, C. Bulbarela-Sampieri, S. Furlan, A. Maisons, M.-P. Chapot-Chartier, M. Erkelenz, P. Mervelet, P. Noirot, D. Frees, O.P. Kuipers, et al., J. Biol. Chem. 282 (2007) 19342–19354.
[46] J.T. Weadge, J.M. Pfeffer, A.J. Clarke, BMC Microbiol. 5 (2005) 49.
[47] J.P. Dillard, K.T. Hackett, Infect. Immun. 73 (2005) 5697–5705.
[48] P.J. Moynihan, A.J. Clarke, J. Biol. Chem. 285 (2010) 13264–13273.
[49] W. Brumfitt, A.C. Wardlaw, J.T. Park, Nature 181 (1958) 1783–1784.
[50] P.J. Moynihan, A.J. Clarke, Anal. Biochem. 439 (2013) 73–79.
[51] J.T. Weadge, A.J. Clarke, Biochemistry 45 (2006) 839–851.
[52] J.M. Pfeffer, J.T. Weadge, A.J. Clarke, J. Biol. Chem. 288 (2013) 2605–2613.
[53] J.-V. Höltje, Microbiol. Mol. Biol. Rev. 62 (1998) 181–203.
[54] T.J. Dougherty, Antimicrob. Agents Chemother. 28 (1985) 90–95.
[55] J.T. Weadge, A.J. Clarke, Biochemistry 46 (2007) 4932–4941.
[56] J.M. Pfeffer, A.J. Clarke, ChemBioChem 13 (2012) 722–731.
[57] C. Miliani, A. Romani, G. Favaro, J. Phys. Org. Chem. 13 (2000) 141–150.
[58] D. Hesek, M. Lee, W. Zhang, B.C. Noll, S. Mobashery, J. Am. Chem. Soc. 131 (2009) 5187–5193.
[59] D. Hesek, M. Lee, K. Morio, S. Mobashery, J. Org. Chem. 69 (2004) 2137–2146.
[60] D.L. Perlstein, Y. Zhang, T.-S. Wang, D.E. Kahne, S. Walker, J. Am. Chem. Soc. 129 (2007) 12674–12675.
[61] T.-J.R. Cheng, M.-T. Sung, H.-Y. Liao, Y.-F. Chang, C.-W. Chen, C.-Y. Huang, L-Y. Chou, Y-D. Wu, Y-H. Chen, Y-S.E. Cheng, C-H. Wong, C. Ma, W-C. Cheng, Proc. Natl. Acad. Sci. USA 105 (2008) 431–436.
[62] C. Fraipont, F. Sapunaric, A. Zervosen, G. Auger, B. Devreese, T. Lioux, D. Blanot, D. Mengin-Lecreulx, P. Herdewijn, J. Van Beeumen, J.-M. Frere, M. Nguyen-Disteche, Biochemistry 45 (2006) 4007–4013.
[63] N. Helassa, W. Vollmer, E. Breukink, T. Vernet, A. Zapun, Febs J. 279 (2012) 2071–2081.
[64] T.-S.A. Wang, S.A. Manning, S. Walker, D. Kahne, J. Am. Chem. Soc. 130 (2008) 14068–14069.
[65] M.D. Lebar, T.J. Lupoli, H. Tsukamoto, J.M. May, S. Walker, D. Kahne, J. Am. Chem. Soc. 135 (2013) 4632–4635.
[66] M. Banzhaf, B. van den Berg van Saparoea, M. Terrak, C. Fraipont, A. Egan, J. Philippe, A. Zapun, E. Breukink, M. Nguyen-Distèche, T. den Blaauwen, W. Vollmer, Mol. Microbiol. 85 (2012) 179–194.
[67] U. Bertsche, E. Breukink, T. Kast, W. Vollmer, J. Biol. Chem. 280 (2005) 38096–38101.
[68] P. Born, E. Breukink, W. Vollmer, J. Biol. Chem. 281 (2006) 26985–26993.
[69] A. Zapun, J. Philippe, K.A. Abrahams, L. Signor, D.I. Roper, E. Breukink, T. Vernet, ACS Chem. Biol. 8 (2013) 2688–2696.