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Mechanism of Action of Peptidoglycan *O*-Acetyltransferase B Involves a Ser-His-Asp Catalytic Triad

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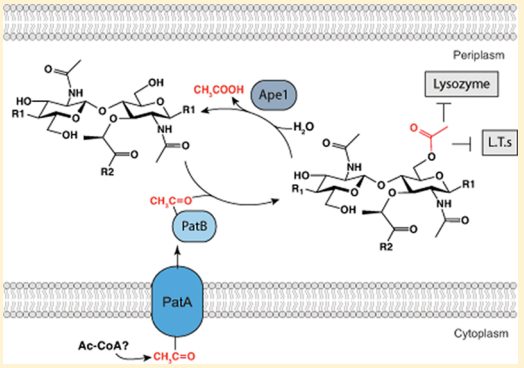
Supporting Information

ABSTRACT: The O-acetylation of the essential cell wall polymer peptidoglycan is essential in many bacteria for their integrity and survival, and it is catalyzed by peptidoglycan *O*-acetlytransferase B (PatB). Using PatB from *Neisseria gonorrhoeae* as the model, we have shown previously that the enzyme has specificity for polymeric muropeptides that possess tri- and tetrapeptide stems and that rates of reaction increase with increasing degrees of polymerization. Here, we present the catalytic mechanism of action of PatB, the first to be described for an *O*-acetyltransferase of any bacterial exopolysaccharide. The influence of pH on PatB activity was investigated, and pK_a values of 6.4−6.45 and 6.25−6.35 for the enzyme−substrate complex (k_{cat} vs pH) and the free enzyme (k_{cat}·K_M^{-1} vs pH), respectively, were determined for the respective cosubstrates. The enzyme is partially inactivated by sulfonyl fluorides but not by EDTA, suggesting the participation of a serine residue in its catalytic mechanism. Alignment of the known and hypothetical PatB amino acid sequences identified Ser133, Asp302, and His305 as three invariant amino acid residues that could potentially serve as a catalytic triad. Replacement of Asp302 with Ala resulted in an enzyme with less than 20% residual activity, whereas activity was barely detectable with (His305 → Ala)PatB and (Ser133 → Ala)PatB was totally inactive. The reaction intermediate of the transferase reaction involving acetyl- and propionyl-acyl donors was trapped on both the wild-type and (Asp302 → Ala) enzymes and LC-MS/MS analysis of tryptic peptides identified Ser133 as the catalytic nucleophile. A transacetylase mechanism is proposed based on the mechanism of action of serine esterases.



Many of the enzymes involved in the metabolism of the essential bacterial cell wall polymer peptidoglycan (PG) have been validated as important antibacterial targets. The most obvious example of these are the penicillin-binding proteins which are the target of the numerous β-lactam antibiotics currently in use by clinicians. The β-lactams mimic the substrate of the transpeptidation reaction responsible for PG cross-linking, and they function as mechanism-based inhibitors of a reaction which features a nucleophilic Ser. The development of more efficacious β-lactam antibiotics has, in large part, been supported by biochemical studies of the catalytic mechanism of these enzymes partnered with structural biology. However, current therapies are failing because of increasing issues of antimicrobial resistance thus requiring the development of new classes of antimicrobials with novel targets. One such target may be the enzymes involved in the O-acetylation of PG.

O-Acetylation of PG occurs predominantly at the C-6 hydroxyl moiety of *N*-acetylmuramoyl (MurNAc) residues in both Gram-positive and Gram-negative bacteria, and it has been identified in numerous pathogens, such as *Staphylococcus aureus, Bacillus anthracis*, all species of *Enterococcus*, species of *Campylobacter*, *Listeria monocytogenes*, *Neisseria gonorrhoeae*, and *N. meningitidis* (reviewed in ref 1). O-Acetylation of *N*-acetylglucosaminyl (GlcNAc) residues also has been reported recently in species of *Lactobacillus*.[2] The O-acetylation of MurNAc specifically protects the glycosidic bond in peptidoglycan (PG) linking the residue to GlcNAc from the activity of host-derived lysozyme by sterically hindering binding of the enzyme to its substrate.[1,3] This is clearly demonstrated in *S. aureus* where O-acetylation renders the organism almost completely resistant to lysozyme.[4] With Gram-negative bacteria, the modification blocks the activity of lytic transglycosylases (reviewed in ref 1), endogenous bacterial enzymes required for PG biosynthesis and turnover, and the insertion of cell-wall spanning structures such as flagella and secretory systems (reviewed in ref 3). The lytic transglycosylases cleave the same glycosidic linkage as lysozyme, but, not acting as a hydrolase, they require a free C-6 hydroxyl group to form their characteristic 1,6-anhydroMurNAc product.[5] This protection of the C-6 hydroxyl has been proposed to provide substrate-level control of lytic transglycosylase activity[1,2] to regulate, for example, glycan chain-length in *N. meningitidis*.[6]

PG O-acetylation is a maturation event, occurring after the transglycosylation and transpeptidation of PG precursors into the extracellular sacculus (reviewed in ref 7). In Gram-positive organisms, *O*-acetyltransferase (Oat) A has been identified

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through genetic screens as the enzyme responsible for the O-acetylation of MurNAc residues,[4,8] while a paralog, OatB, is responsible for the O-acetylation of GlcNAc in *Lactobacillus plantarum*.[2,4] These enzymes are predicted to be bimodal, possessing an N-terminal transmembrane region responsible for translocation of acetyl groups across the cytoplasmic membrane, presumably from acetyl-CoA, while an extracellular C-terminal domain is proposed to catalyze the transacetylase activity.[7] With Gram-negative bacteria, two proteins, PatA and PatB, perform the modification.[9,10] PatA is predicted to be a large transmembrane protein with similarity to the membrane-bound *O*-acetyltransferase (MBOAT) class of enzymes. PatA is proposed to transfer the acetyl to the periplasmic face of the cytoplasmic membrane where the peripheral membrane *O*-acetyltransferase PatB (E.C. 3.2.1.x) transfers it to PG.[9,10] This pathway has been validated experimentally with PatB from *N. gonorrhoeae* as the model.[11] Interestingly, species of *Bacillus*, including *B. anthracis*, produce both the Oat and PatA/B systems for PG O-acetylation.[12]

Recently, we reported the first in vitro assay developed for PG *O*-acetyltransferases which exploits their ability to utilize both chitooligo-saccharides as acceptors and chromogenic acetylesterase substrates as acetyl donors.[13] Using this assay, we then presented the first biochemical analysis of any PG *O*-acetyltransferase, using PatB from *N. gonorrhoeae* as the model.[14] The enzyme was found to have specificity for polymeric muropeptides that possess tri- and tetrapeptide stems, and with simple chitooligosaccharides as substrates, rates of reaction increase with increasing degrees of polymerization.

A genomic study[9] predicted the *patB* gene of *N. gonorrhoeae* to encode an SGNH/GDSL-family esterase with similarity to authentic *O*-acetylPG esterase 1 (Ape1);[15] PatB shares 20.6% amino acid identity and 58.5% similarity with Ape1. Alignment of the two *N. gonorrhoeae* protein sequences reveals that the identity between them includes the consensus motifs of Ape that involve invariant Ser, His, and Asp residues which have been confirmed experimentally to function as a catalytic triad in the classical serine esterase mechanism of action.[16,17] Whereas PatB has been proven to function as an *O*-acetyltransferase, it does exhibit weak esterase activity.[11,14] Given this, we postulated that PatB uses a catalytic triad of Ser, His, and Asp to perform the acetyltransferase reaction in a mechanism analogous to that of the classical serine esterases. The protein engineering and enzyme kinetic experiments presented herein provide direct evidence in support of this postulate and identify Ser133, His305, and Asp302 as the catalytic triad essential for *O*-acetyltransferase activity.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents. Acrylamide and glycerol were purchased from Fisher Scientific (Nepean, ON), and chitooligosaccharides were products of Carbosynth (Berkshire, UK). All growth media were obtained from Difco Laboratories (Detroit, MI). Ni^{2+}-Nitrilotriacetic acid (Ni^{2+}NTA) agarose was a product of Qiagen (Valencia, CA), Source Q was purchased from GE Healthcare (Piscataway, NJ), graphitized carbon solid phase extraction columns (Carbograph SPE) were products of Grace Canada, Inc. (Ajax, ON), and Hypercarb Porous Graphitized Carbon (PGC) columns were supplied by Thermo Electron Corp. (Rockford, IL). Unless otherwise stated, all other chemicals and reagents were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON).

Bacterial Strains and Culture Conditions. The bacterial strains used in this study and their genotypes are presented in Supplemental Table 1, Supporting Information. All plasmids constructed (Supplemental Table 1) were screened and maintained in *Escherichia coli* DH5α. *E. coli* BL21*-λDE3 was used for the overproduction of recombinant proteins. Cells were always freshly transformed with the desired expression plasmid and grown in Super Broth (5 g of sodium chloride, 20 g of yeast extract, and 32 g of tryptone) at 37 °C with agitation. Cultures were supplemented with chloramphenicol (35 mg·L^{-1}), ampicillin (100 mg·L^{-1}) and kanamycin (50 mg·L^{-1}) where appropriate.

Site-Directed Mutagenesis. Site-directed mutagenesis of *patB* was conducted using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) with the appropriate primers (Supplemental Table 2, Supporting Information) and pACPM33[14] as the template. Following PCR, *Dpn* I was added to the reaction mixture to remove the original methylated template. The remaining plasmids in the reaction mixture were then used to transform *E. coli* DH5α, and the resulting clones were screened for the correct alterations by DNA sequencing (Guelph Molecular Supercenter, University of Guelph, ON).

Production and Purification of $PatB_{SUMO}$ and Its Engineered Forms. *E. coli* BL21*-λDE3 was transformed with the appropriate plasmid (Supplemental Table 1), cultured in Super Broth, and induced for overproduction of the recombinant forms of PatB presented in this study as described previously.[14] Likewise, these various PatB forms were isolated and purified to >95% homogeneity, as judged by SDS PAGE and MS analysis, using a combination of affinity chromatography on Ni^{2+}-NTA-agarose and anion-exchange chromatography on SourceQ as recently described.[14] As before, proteins recovered by affinity chromatography were dialyzed exhaustively at 4 °C against 20 mM sodium phosphate buffer, pH 8.0 containing 100 mM NaCl and 50 mM L-arginine, and then against the same buffer lacking the NaCl, followed by the same buffer but lacking the NaCl and arginine to keep the PatB forms soluble. Also, precaution was taken to use fresh chromatography media for each purification to preclude cross contamination of the different PatB forms. The purified protein was used immediately.

Circular Dichroism Spectroscopy. Circular dichroism spectroscopy was used to assess the secondary structure of wild-type PatB and its variant forms involving site-specific amino acid replacements. After purification, proteins in 20 mM sodium phosphate buffer, pH 8, containing ~100 mM NaCl were dialyzed stepwise (to prevent precipitation of protein) into 10 mM sodium phosphate buffer, pH 7.0. Each successive dialysis step was carried out in 1 L of buffer for 4 h and involved reducing NaCl concentrations by 50 mM and pH by 0.25 units. Dialysis in the final buffer was repeated twice to ensure complete removal of NaCl. Far UV CD spectra were collected using a Jasco J-801 spectrapolarimeter as described previously.[16,17] Each spectrum was collected as an average of six data accumulations, and it was analyzed using the DichroWeb server with the Selcon3 program and protein reference set 4.[18,19]

Steady-State Kinetics. Routine kinetic assays of the *O*-acetyltransferase activity catalyzed by $PatB_{SUMO}$ and its engineered forms were conducted using the chromogenic assay[13] with enzyme (1.0 or 3.0 μM) in 50 mM sodium phosphate buffer, pH 7 at 37 °C with 4 mM *p*NP-Ac (in 5% ethanol, final volume) and 1 mM chitotetraose or chitopentaose as cosubstrates. Michaelis–Menten parameters (k_{cat} and

K_M) were determined by nonlinear regression analysis of plots of initial velocity as a function of substrate concentration. The effect of pH on $PatB_{SUMO}$ activity was investigated using 30 mM sodium phosphate-citrate-borate buffer at pH 5−8. In all experiments, $n \geq 3$.

Chemical Modification of Predicted Catalytic Residues. Chemical modification of carboxylic side groups was performed by reaction of 1 μM PatB in 50 mM Bis-Tris-Propane buffer, pH 6.0 with 5 mM 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide for 18 h at 4 °C. Diethylpyrocarbonate was used to modify imidazolium rings of His residues by addition to protein at 5 mM at pH 8 in 50 mM sodium phosphate buffer for 30 min at 25 °C. Finally, the modification of the putative catalytic Ser residue was attempted by the addition of 0.5 mM phenylmethylsulfonyl fluoride (PMSF) to 1 μM PatB in 50 mM sodium phosphate buffer, pH 6.5 and incubated for 30 min at 25 °C. A control reaction lacking the modifying compound in each case was incubated under similar conditions and activity of each enzyme mixture was compared to assays including the appropriate controls.

Trapping of the Covalent-Enzyme Intermediate. An acetyltransferase reaction was initiated by the addition of 1 μM PatB in 1 mL of 50 mM sodium phosphate buffer, pH 6.5 with 4 mM *p*NP-Ac and 1 mM chitopentaose. After 1 min, the reaction was stopped by the addition of 5 mL of cold acetone. The precipitated protein was washed thrice with cold acetone, resuspended in 100 μL of 25 mM sodium bicarbonate buffer, pH 7.5, and digested with 0.1 μg of proteomic-grade trypsin for 18 h at 37 °C. The resulting peptides were analyzed by LC-MS.

LC-MS/MS Analysis of Tryptic Peptides. LC-MS/MS analyses were performed on an Agilent 1200 HPLC liquid chromatograph interfaced with an Agilent UHD 6530 Q-TOF mass spectrometer. Separation of the peptides was achieved by reverse-phase HPLC using a C18 column (Agilent AdvanceBio Peptide Map, 100 mm × 2.1 mm 2.7 μm) previously equilibrated with 2% acetonitrile in 0.1% formic acid at a flow rate of 0.2 mL·min^{-1}. Following injection of samples, a linear gradient to 45% acetonitrile in 0.1% formic acid was applied over 40 min and then to 55% acetonitrile over 10 min. The column was washed with 95% acetonitrile in 0.1% formic acid for 5 min before re-equilibration in starting solvent. The mass-to-charge ratio was scanned across the m/z range of 300−2000 m/z in 4 GHz (extended dynamic range positive-ion auto MS/MS mode). Three precursor ions per cycle were selected for fragmentation. The instrument was calibrated with the ESI TuneMix (Agilent).

Raw data files were loaded directly into PEAKS 7 software (Bioinformatics Solutions Inc.) where the data were refined and subjected to deNovo sequencing and database searching. The following modifications were considered within the search parameters: Met oxidation, and acetylation of Thr, Ser, Cys, Tyr, and His residues. The data were searched against the PatB sequence. The tolerance values used were 10 ppm for parent ions and 0.5 Da for fragment ions.

Other Analytical Techniques. Protein concentrations were determined using a bicinchoninic acid assay (Pierce, Rockford, IL). SDS-PAGE on 12% acrylamide gels was conducted by the method of Laemmli[20] with Coommassie Brilliant Blue staining and Western immunoblot analysis as previously described.[11] All MS analyses were conducted using instruments at the Mass Spectrometry Facility of the University of Guelph.

RESULTS

Production and Purification of PatB. $PatB_{SUMO}$ was produced in *E. coli* BL21*-λDE3 harboring pACPM33, a form of PatB lacking its 36 amino acid periplasmic-localizing signal sequence but possessing an N-terminal fusion to His_6−SUMO.[14] The purified protein was determined to have a specific activity of 0.51 ± 0.01 μmol·min^{-1}·mg^{-1} as an *O*-acetyltransferase using *p*NP-Ac and chitopentaose as cosubstrates at pH 7.0. These values are very similar to those obtained previously for $PatB_{SUMO}$.[14] This fusion form of PatB has to be used for biochemical studies because removal of SUMO results in rapid loss of activity and degradation of the enzyme.[14]

Effect of pH on $PatB_{SUMO}$ Activity. The pH−activity profile of $PatB_{SUMO}$ acting on *p*NP-Ac as substrate is relatively broad and bell-shaped,[14] suggesting the participation of at least two ionizable groups in the enzyme's mechanism of action. To further assess this pH dependence, the Michaelis−Menten parameters for the $PatB_{SUMO}$-catalyzed transfer of acetyl from *p*NP-Ac to chitotetraose were determined at different pH values ranging from 6 to 8.0; the concentration of one cosubstrate was maintained at saturating levels while that of the other was varied. Limitations of enzyme stability and assay sensitivity at lower pH values, and substrate stability at higher pH, precluded a broader analysis with appropriate reliability. Dixon plots[21] prepared from these data (Figure 1) indicated the

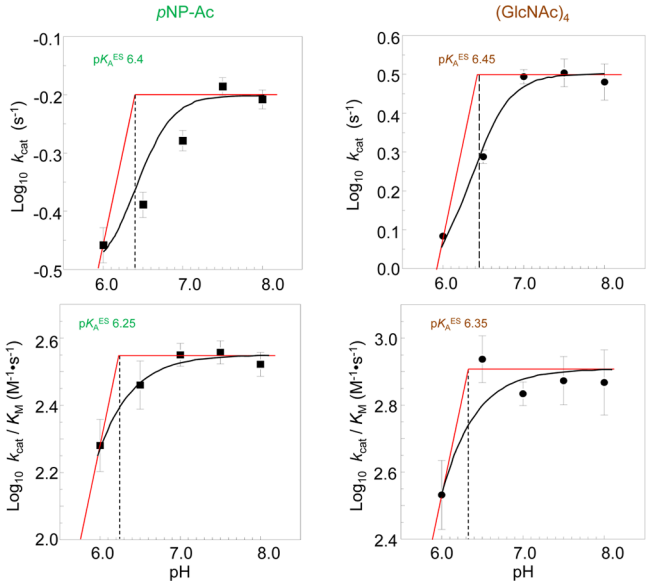

Figure 1. Dependence of $PatB_{SUMO}$ Michaelis−Menten parameters on pH. The Michaelis−Menten parameters k_{cat} and $k_{cat}{\cdot}K_M^{-1}$ were determined for (■) *p*NP-Ac and (●) chitotetraose in 30 mM sodium phosphate-citrate-borate buffer at pH 6−8 where the respective cosubstrate was maintained at saturating levels. The theoretical Dixon lines (red lines: slopes of 1.0, and estimated k_{cat} and $k_{cat}{\cdot}K_M^{-1}$ maxima (slopes of 0)) were plotted to provide estimates of pK_A values (dashed lines) as indicated. The error bars denote SD ($n \geq 3$).

dependency of the reaction on the ionization of at least one group. The plot of k_{cat} vspH provided estimated pK_A^{ES} values of 6.4−6.45 for the essential ionizable group in the enzyme−substrate complexes, while values of 6.25−6.35 were obtained from the $k_{cat}{\cdot}K_M^{-1}$ vs pH plot for pK_A^E of a group on the free enzyme. These pK_A values are very similar to those obtained with *N. gonorrhoeae* Ape1[16] and, as with the esterase, the

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identity of the ionizable group in PatB is likely the imidazolium of a histidyl residue.

Order of the Reaction Catalyzed by PatB. Cleland's rules[22] were applied to assess the order of the two-substrate, two-product reaction catalyzed by PatB. Thus, $PatB_{SUMO}$ was assayed in 50 mM sodium phosphate buffer, pH 7.0, with cosubstrates at concentrations bracketing the K_M for each (1, 2, 3, and 4 mM for *p*NP-Ac and 0.5, 1, 2, and 2.5 mM for chitopentaose). Double reciprocal plots of initial velocity *versus* (i) chitopentaose at fixed concentrations of *p*NP-Ac and (ii) *p*NP-Ac at fixed concentrations of chitopentaose were prepared (Figure 2). Analysis of the resulting data using a one-way

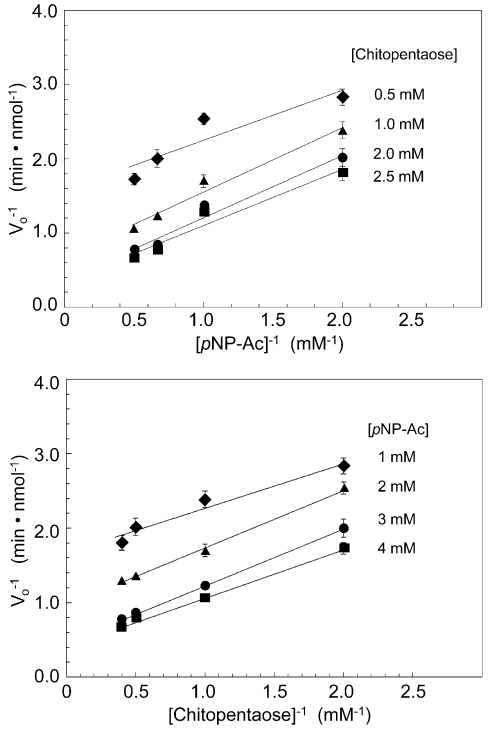

Figure 2. Determination of reaction order of $PatB_{SUMO}$ by kinetic analysis. Lineweaver–Burk plots of initial rates of reaction of 1 μM $PatB_{SUMO}$ in 50 mM sodium phosphate buffer, pH 7.0, with *p*NP-Ac as acetyl donor and chitopentaose as acceptor at the concentrations shown.

ANOVA determined that the slopes of the respective curves were not significantly different from one another ($F(3,8)$ = 1.145, p = 0.3883). A postanalysis Tukey's test was used to compare each pair of lines in the group, and none were found to be significantly different from each other ($p > 0.1$). These data thus suggest that the reaction pathway catalyzed by PatB follows a double-displacement ping-pong, bibi mechanism.

Identification of Catalytic Residues. On the basis of sequence alignments and homology modeling, we had earlier proposed that *N. gonorrhoeae* PatB is a member of SGNH hydrolase superfamily,[9] and thus it would adopt the canonical α/β fold of these proteins. Consequently, it was surprising to later discover that PatB is not a hydrolase (esterase), but rather it functions as a transacetylase.[11] An updated BLAST search for hypothetical homologues of PatB added 11 amino acid sequences to the alignment which now involves a total of 52 known and hypothetical proteins (Figure S1, Supporting Information). This list largely involves species of the Proteobacteria (alpha, beta, gamma, delta, epsilon), although a few representatives of the Spirochaetes, Acidobacteriaceae, and Actinobacteria are also included. Only 13 residues of these aligned sequences appear to be invariant, but these still include the GDS and DxxH motifs that comprise the signature consensus motifs of the GDSL subset of the SGNH hydrolase superfamily of hydrolases (reviewed in ref 23).

The three-dimensional structure of any PG *O*-acetyltransferase (or PG *O*-acetylesterase) remains unknown despite concerted efforts to produce crystals that diffract with appropriate resolution. Consequently, in an earlier study[11] we generated a predicted partial structure of PatB which was based on the known structure of acyl-CoA thioesterase I (PDB: 1JRL). With many more structures made available over the past three years, we refined this predicted structure using Phyre2. This involved subjecting the entire amino acid sequence of *N. gonorrhoeae* PatB for prediction, and isoamyl acetate-hydrolyzing esterase from *Sacchromyces cerevisiae* (PDB: 3MIL) was selected by the program to serve as the template. Importantly, this prediction yielded a fidelity score 99.9% with 57% coverage over 188 (C-terminal) residues of PatB. The prediction included Ser63-Pro327 of PatB and, being modeled on an SGNH hydrolase, the structure adopts an α/β-hydrolase fold that contains a central four-stranded parallel twisted β-sheet flanked by five α-helices (Figure 3). This prediction includes

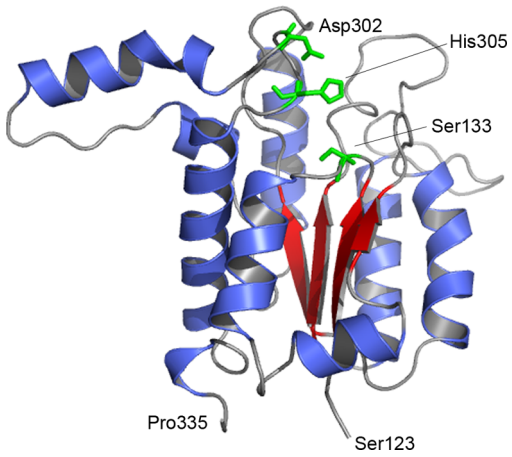

Figure 3. Predicted three-dimensional structure of the *N. gonorrhoeae* PatB. Residues 123–335 of PatB were threaded by Phyre2 onto the SGNH hydrolase isoamyl acetate-hydrolyzing esterase from *Sacchromyces cerevisiae* (PDB 3MIL). The positions of the putative catalytic Ser133, Asp-302, and His-305 residues are appropriately aligned to serve as the catalytic center of the enzyme.

Ser133 in addition to Asp302 and His305 of the conserved motifs, and they are positioned in an appropriate alignment within a pocket of the enzyme to presumably function in a reverse reaction that is typical of SGNH esterases. The web-based server DaliLite was used to compare the predicted PatB structure to the only solved structure of an authentic *O*-acetyltransferase of the α/β-hydrolase superfamily of serine enzymes, homoserine transacetylaase (PDB, 2B61). The Z-score for this comparison was 2.5 involving 119 equiv residues and the root-mean-square deviation of C-alphas was 3.5, indicating some general similarity between the enzymes.

In a preliminary attempt to determine the importance of the invariant Ser, His, and Asp residues, we sought to determine the impact of chemical modification. Despite repeated attempts, a decrease in activity of the enzyme was not observed after

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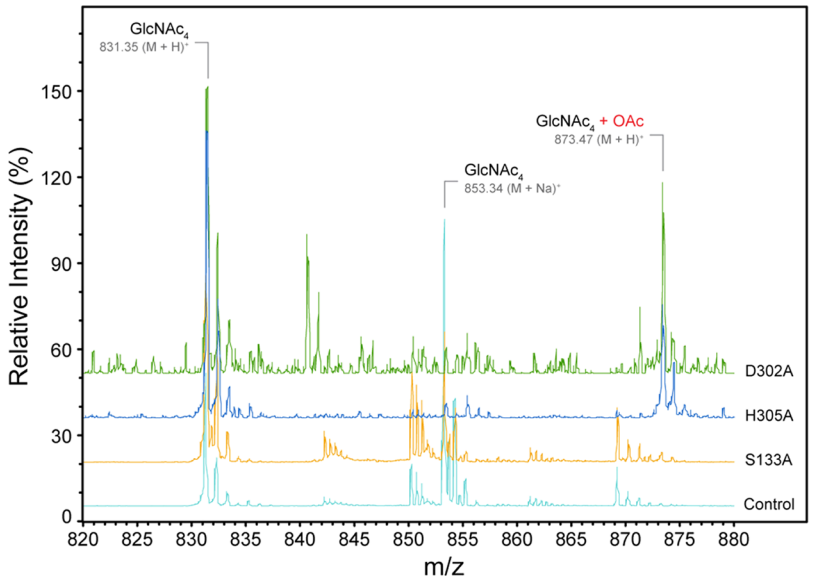

Figure 4. ESI-Ion Trap MS analysis of reaction products of $PatB_{SUMO}$ variants. Respective enzymes (3 μM) in 50 mM sodium phosphate buffer, pH 7.0, were incubated with 4 mM *p*NP-Ac and 1 mM chitotetraose for 15 min. Reaction products were desalted by chromatography on graphitized carbon prior to ESI-Ion Trap MS.

chemical modification of Asp/Glu and His residues through the addition of either carbodimide or diethylpyrocarbonate, respectively. This lack of inhibition by the two reagents could be due to either the absence of the targeted side chains at the catalytic site of PatB or the inability of the modifying compounds to access the active site pocket. However, addition of 0.5 mM PMSF, a classic serine protease/esterase/lipase inhibitor, resulted in 38% inactivation. Unfortunately, increased PMSF concentrations resulted in the formation of precipitates which thus precluded determination of the full extent of PMSF inactivation of the enzyme. A catalytic Cys is not possible in this enzyme because PatB does not contain any Cys residues.

To confirm the participation and identity of the residues essential to the mechanism of action of PatB, site-specific amino acid substitutions were made to each of the predicted catalytic residues by site-directed mutagenesis. All three mutant genes encoding the Ser133 → Ala, His305 → Ala, and Asp302 → Ala forms of PatB were overexpressed in *E. coli* BL21* transformants, as confirmed by SDS-PAGE and Western blot analysis with an anti-His_6 antibody (data not shown). Each overproduced enzyme was purified by the same method employed for wild-type PatB taking the precaution to use new chromatography media for each purification thereby precluding any potential contamination of the enzyme forms with each other. This protocol served to provide the enzymes in apparent homogeneous forms, and yields of the variant enzymes were comparable to those obtained with wild-type PatB. Each of the Ser133 → Ala, His305 → Ala, and Asp302 → Ala PatB variants had consistent secondary structural elements as determined by CD spectroscopy (data not shown) suggesting that the amino acid replacements did not cause significant structural alterations to the enzyme.

Kinetic analysis of the enzymes possessing replacements of each of the catalytic residues revealed substantial defects in activity. Replacement of the catalytic Ser or His residues resulted in a complete abrogation of detectable activity using the chromogenic assay. Replacement of the predicted catalytic Asp, however, resulted in a decrease in specific activity by 80%. It was not possible to determine the Michaelis–Menten parameters for (Asp302 → Ala)PatB due to its low residual activity and the limitations of the assay. Further analysis of reaction products by ESI-Ion Trap MS, however, revealed that the His and Asp replacement generated some detectable product, while no product was detected in reactions with the Ser replacement (Figure 4).

Trapping of the Covalent-Enzyme Intermediate. Nucleophilic catalysis through a double-displacement ping-pong bibi reaction pathway requires the formation of a covalent intermediate, but in the context of the active enzyme in an aqueous solvent, it would be unstable and short-lived, especially considering the apparent esterase activity of the enzyme. Acetyl-Ser, in contrast, is relatively stable in aqueous solutions, and so we reasoned that we could trap the covalent enzyme intermediate by simultaneously excluding water from the active site and denaturing the enzyme during the course of reactions. Thus, after 1 min of incubation of wild-type PatB and its Asp302 → Ala and His305 → Ala variants with *p*NP-Ac, each in the absence and presence of chitopentaose, the reaction was quenched by the addition of cold acetone, and the precipitated proteins were subjected to trypsin digestion and LC-MS analysis. The mass spectra of the separated tryptic peptides were compared to predicted *in silico* digestions of PatB. Approximately 98% coverage of PatB was achieved with high confidence with each of these analyses. In addition to peptides containing the putative catalytic Ser133 residue, similar peptides with a mass shift consistent with acetylation were also identified with both wild-type PatB and the Asp302 → Ala variant. Analysis of MS/MS data from these peptides localized the additional 42.01 mass of an acetyl adduct to the Ser residue with high confidence (Figure 5; Table 1). A comparison of the total ion chromatograms for the modified and unmodified peptides indicated that approximately 25% of the total population of tryptic peptides that contained the catalytic Ser were acetylated. The acetylated forms of these peptides were not detected with the His305 → Ala variant incubated with *p*NP-Ac. Likewise, control reactions lacking *p*NP-Ac showed no evidence of the acetyl modification.

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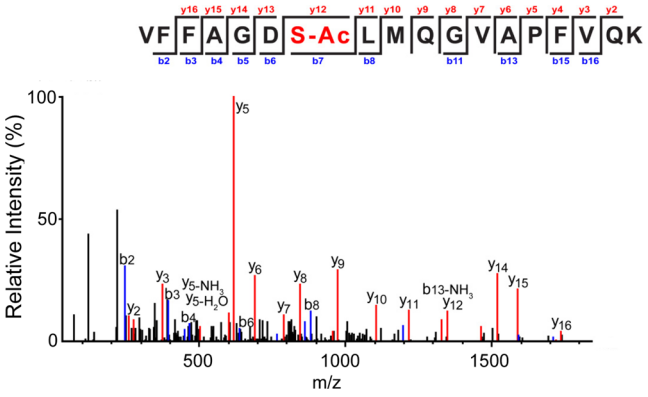

Figure 5. Identification of the acetyl-PatB$_{SUMO}$ intermediate by Q-ToF MS/MS. PatB$_{SUMO}$ (1 μM) in 50 mM sodium phosphate buffer, pH 7.0 was treated with 1 mM *p*NP-Ac and 1 mM chitopentaose. After 1 min incubation, the reaction was quenched with cold acetone. Precipitated protein was washed thrice with cold acetone and then digested with trypsin prior to MS/MS analysis. Inset: Identification of the y and b ions (see Table 1 for masses of identified ions).

Table 1. Identification of *O*-acetyl-Ser Containing Peptides by LC- Q-TOF MS/MS[a]

#	b	b-H_2O	b-NH_3	Sequence	y	y-H_2O	y-NH_3	#
1	100.08	82.07	83.05	V				
2	247.14	229.13	230.11	F	1883.94	1865.93	1866.91	17
3	394.21	376.20	377.18	F	1736.85	1718.84	1719.82	16
4	465.25	447.24	448.22	A	1589.79	1571.78	1572.76	15
5	522.26	504.25	505.23	G	1518.77	1500.76	1501.74	14
6	637.29	619.28	620.26	D	1461.73	1443.72	1444.70	13
7	766.34	748.33	749.31	S (+42.01)	1346.70	1328.69	1329.67	12
8	879.41	861.40	862.38	L	1217.68	1199.67	1200.65	11
9	1010.47	992.46	993.44	M	1104.58	1086.57	1087.55	10
10	1138.52	1120.51	1121.49	Q	973.55	955.54	956.52	9
11	1195.54	1177.53	1178.51	G	845.48	827.47	828.45	8
12	1294.61	1276.60	1277.58	V	788.46	770.45	771.43	7
13	1365.65	1347.64	1348.62	A	689.40	671.39	672.37	6
14	1462.7	1444.69	1445.67	P	618.36	600.35	601.33	5
15	1609.77	1591.76	1592.74	F	521.30	503.29	504.27	4
16	1708.85	1690.84	1691.82	V	374.24	356.23	357.21	3
17	1836.9	1818.89	1819.87	Q	275.17	257.16	258.14	2
				K	147.11	129.10	130.08	1

[a]Identified b ions and y ions corresponding to Figure 5 are in blue and red, respectively. All ions were identified with less than 0.1 Da error.

The experiment described above was repeated with wild-type PatB except *p*NP-Ac was replaced with propionyl-CoA as the acyl-donor. Only the tryptic peptides containing the putative catalytic Ser133 were found to possess the additional mass of 56.06 g/mol of the expected propionyl adduct, and MS/MS based sequencing (Figure 6, Table 2) confirmed it was covalently bound to Ser133.

DISCUSSION

We report herein the first understanding of the mechanistic basis of PatB activity, a major virulence determinant found in numerous bacterial pathogens, both Gram-positive and Gram-negative[6,12,24,25] and a potential new target for antibacterial therapy. Our elucidation of the catalytic pathway of PatB involving a catalytic triad of Ser, His, and Asp residues provides valuable insight for the search for, and development of, inhibitors that may serve as leads for the generation of new classes of antibiotics.

It is presumed that, in vivo, PatA functions to translocate acetyl groups from cytoplasmic pools through the cytoplasmic membrane to the periplasm for its transfer to PG by PatB.[9,11]

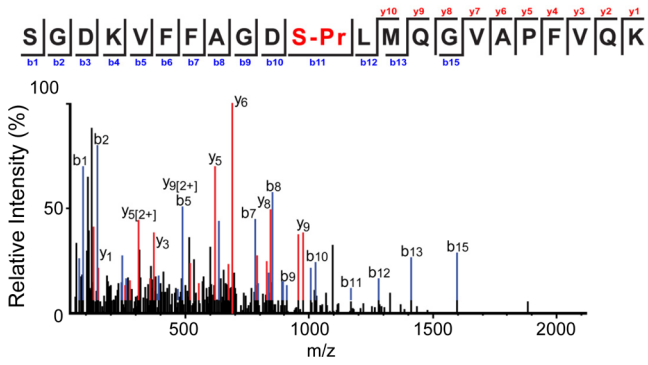

Figure 6. Identification of the propionyl-PatB intermediate by Q-ToF MS/MS. PatB$_{SUMO}$ (1 μM) in 50 mM sodium phosphate buffer, pH 7.0 was treated with 1 mM propionyl-CoA and 1 mM chitopentaose. After 1 min incubation, the reaction was quenched with cold acetone, and precipitated protein was digested with trypsin prior to MS/MS analysis. Inset: Identification of the y and b ions (see Table 2 for masses of identified ions).

Whereas replacement of PatA in vitro with a small chromogenic acetyl donor, and PG with chito-oligosaccharides, is unlikely to yield physiologically relevant kinetic parameters, it does provide the opportunity to investigate the catalytic mechanism of the enzyme. Coupled with site-specific replacements of potential catalytic residues, this kinetic analysis of PatB clearly demonstrated that PG O-acetylation proceeds by nucleophilic catalysis involving a catalytic triad of Ser-His-Asp residues that apply a ping-pong bisubstrate biproduct mechanism. Several lines of evidence support these conclusions: (i) Cleland's rules[21] state that the pattern of parallel lines generated by double reciprocal plots of enzyme velocity versus chitopentaose concentration at changing concentrations of acetyl donor *p*NP-Ac obtained with the kinetic analysis of PatB would result from a ping-pong bibi mechanistic pathway; (ii) the trend of decreasing catalytic activity associated with Asp302Ala (20% residual activity), His305Ala (detectable only by ESI-MS analysis of reaction products), and Ser133Ala (no detectable activity) variants of PatB would be expected for their participation in a proton relay network of a catalytic triad;[26] (iii) acetyl was trapped on the putative catalytic Ser133 residue of both the wild-type enzyme and only the Asp302 → Ala variant confirming the existence of the expected covalent acyl-enzyme intermediate; and (iv) a propionyl-Ser133 adduct was isolated when PatB was incubated with propionyl-CoA confirming the identity of Ser133 as a catalytic residue and not simply a Ser residue involved in post-translational modification as can occur with acetylation. Our approach to trapping the acyl-enzyme intermediate, involving both acetyl and propionyl adducts allowed direct determination of the amino acid residue involved. In contrast to radiolabeling experiments, this methodology can yield quantitative and direct observation of a covalent-catalytic amino acid intermediate. This experimental approach would be broadly applicable to enzymatic systems where the identification of a catalytic residue may not be clear without the use of inhibitors.

On the basis of the preceding data, we propose the following double-displacement mechanism of action for PatB which is predicated on our understanding of the activity of the serine esterase and lipase superfamily of enzymes, including *N. gonnorhoeae* Ape1.[16,17] The binding of substrate is predicted to trigger a proton relay involving the catalytic triad residues Ser133, His305, and Asp302 in which the carboxyl group of

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Table 2. Identification of *O*-Propionyl-Ser Containing Peptides by LC- Q-ToF MS/MS[a]

#	b	b-H_2O	b-NH_3	$b^{(2+)}$	Sequence	y	y-H_2O	y-NH_3	$y^{(2+)}$	#
1	88.04	70.07	71.01	44.52	S					
2	145.06	127.05	128.03	73.03	G	2297.20	2279.19	2280.17	1149.1	21
3	260.11	242.08	243.06	130.54	D	2240.18	2222.17	2223.15	1120.59	20
4	388.17	370.17	371.16	194.59	K	2125.15	2107.14	2108.13	1063.08	19
5	487.25	469.24	470.22	244.13	V	1997.06	1979.05	1980.03	999.03	18
6	634.32	616.31	617.29	317.66	F	1897.99	1879.98	1880.96	949.49	17
7	781.39	763.38	764.36	391.2	F	1750.92	1732.91	1733.89	875.96	16
8	852.41	834.42	835.4	426.71	A	1603.85	1585.84	1586.83	802.43	15
9	909.45	891.44	892.43	455.22	G	1532.82	1514.80	1515.79	766.91	14
10	1024.44	1006.48	1007.45	512.74	D	1475.79	1457.78	1458.77	738.40	13
11	1167.53	1149.56	1150.54	584.28	S(+56.06)	1360.77	1342.76	1343.74	680.88	12
12	1280.60	1262.64	1263.63	640.83	L	1217.67	1199.66	1200.64	609.34	11
13	1411.74	1393.68	1394.67	706.35	M	1104.59	1086.58	1087.56	552.79	10
14	1539.75	1521.74	1522.73	770.38	Q	973.53	955.53	956.49	487.25	9
15	1596.74	1578.76	1579.75	798.89	G	845.48	827.48	828.43	423.24	8
16	1695.84	1677.83	1678.82	848.42	V	788.46	770.46	771.44	394.73	7
17	1766.88	1748.87	1749.85	883.94	A	689.39	671.39	672.37	345.20	6
18	1863.93	1845.92	1846.91	932.47	P	618.36	600.35	601.33	309.69	5
19	2011.00	1992.99	1993.97	1006.00	F	521.31	503.3	504.28	261.15	4
20	2110.07	2092.06	2093.04	1055.53	V	374.24	356.19	357.21	187.62	3
21	2238.13	2220.12	2221.10	1119.56	Q	275.17	257.16	258.14	138.09	2
					K	147.12	129.10	130.09	74.06	1

[a]Identified b ions and y ions corresponding to Figure 6 are in blue and red, respectively. All ions were identified with less than 0.1 Da error.

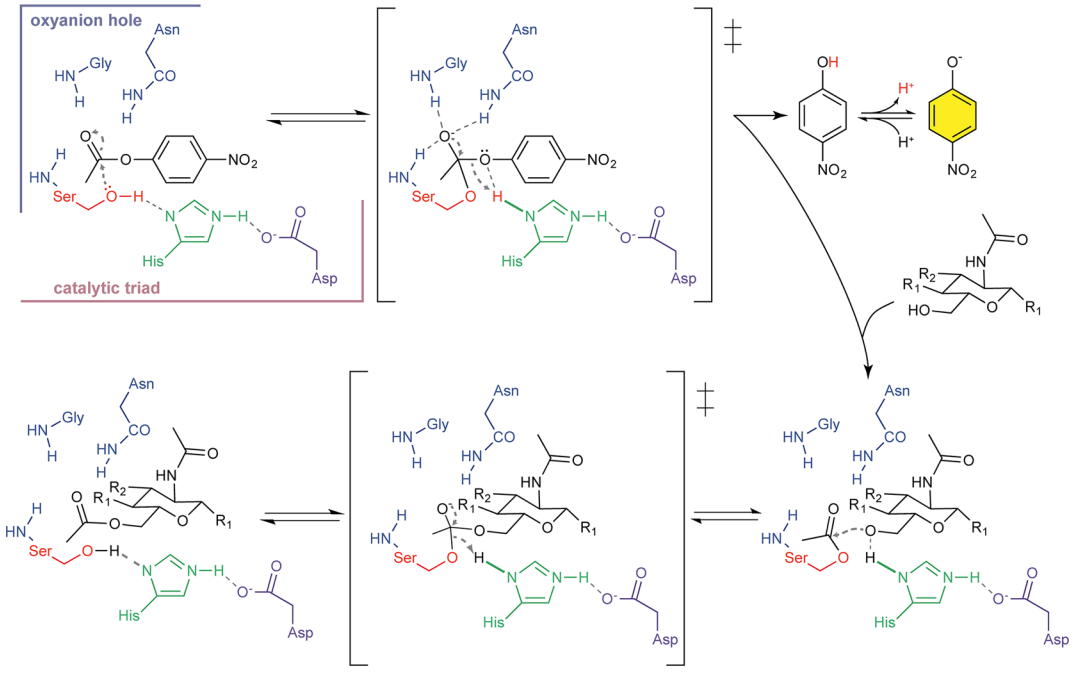

Figure 7. Proposed catalytic mechanism of PG *O*-acetyltransferases. The mechanism is presented with *p*NP-Ac as the acetyl donor but in vivo presumably acetyl-PatA serves this function. R_1 is either a hydroxyl group or a β-1,4 linked sugar; R_2 is either a hydroxyl group of chito-oligosaccharides or a lactyl-peptide moiety of muramoyl-peptides in PG. The nitrophenolate ion is depicted in yellow to signify its detection at 405 nm.

Asp302 forms a salt bridge with the N-δ1 of His305, allowing the N-ϵ2 of its imidazole ring to abstract a proton from Ser133 rendering the latter nucleophilic (Figure 7). This alkoxide of the catalytic Ser would attack the carbonyl carbon of the acetyl donor generating a tetrahedral transition state that would collapse to a covalently bound acyl-enzyme intermediate and the release of the first product while acquiring the proton of the serine hydroxyl from His305. The sugar acceptor would then bind within the active site cleft and, through acid/base catalysis, His305 would abstract the proton of the C-6 hydroxyl group rendering it nucleophilic. Attack by the C-6 alkoxide on the carbonyl center of the acetyl-Ser would lead to the formation of the second tetrahedral transition state. Collapse of the latter would generate the *O*-acetyl sugar product and free the enzyme to repeat the cycle. As with the other serine enzymes, including Ape1,[17] residues at the active center would form an "oxyanion hole" to stabilize the putative transition states. Given that similar motifs containing invariant Ser, His, and Asp residues are found in homologues and paralogs of PatB,[9] including PG *O*-acetyltransferase OatA of Gram-positive bacteria,[4,24] poly-

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sialic acid-specific *O*-acetyltransferase OatC[27] and the putative alginate *O*-acetyltransferases AlgX and AlgJ,[28,29] we further propose that this mechanism of action is a common feature of these enzymes involved in the O-acetylation of PG and other bacterial exopolysaccharides.

As noted above, the reaction mechanism presented is analogous to that known for hydrolases and thioesterases of the α/β-hydrolase superfamily of serine enzymes. However, the significant difference between PatB (and presumably OatA, OatC, AlgX, and AlgJ) and these other enzymes is that it is not a hydrolase where the acetyl group bound to the catalytic Ser of the reaction intermediate is transferred to a water molecule, but instead it is a transacetylase that transfers it to a sugar hydroxyl, in this case the C-6 hydroxyl of muramoyl residues. Whereas transesterification reactions of longer acyl chains are catalyzed by many members of this superfamily, to our knowledge there is only one other transacetylase known, homoserine transacetylase,[30,31] an intracellular enzyme involved in the biosynthesis of aspartate. A relatively high resolution crystal structure of this latter enzyme has been determined, but it is still not clear how water is precluded from the active site to prevent a hydrolytic reaction.[32] As demonstrated here and earlier,[11,13,14] PatB can, in fact, function as a weak esterase in vitro, but it would seem highly unlikely the enzyme performs the hydrolytic reaction in vivo where acetyl groups would be translocated from the cytoplasm by PatA then to be wastefully lost to the periplasm by PatB. Without structural information for PatB, we can only speculate on how the hydrolytic reaction would be prevented, but it is conceivable that water could be excluded from the active site by hydrophobic shielding involving residues on both PatB and PatA, as well as the insoluble PG sacculus, as all three are proposed to combine in a complex for activity in vivo.[7,11] Presumably PatB has a binding site cleft that accommodates both PatA with its bound (translocated) acetyl and a PG chain that is attached to the insoluble sacculus and these associations may shield the acetyl-Ser from water. Moreover, it is possible that the acetyl group is channeled from pools of a cytoplasmic source, presumably acetyl-CoA, through PatA thereby crossing the cytoplasmic membrane to the active site of PatB for its transfer to PG. Such channeling would minimize, if not preclude, the loss of the valuable metabolite during the process of external PG O-acetylation.

As we described recently, PatB in vitro appears to have considerable flexibility to accommodate a variety of pseudo acyl donor and acceptor substrates.[14] Given this, it was a little surprising that the general serine-enzyme inhibitor PMSF only weakly inhibited PatB. With the promiscuity of PatB activity in its ability to act on a variety of different cosubstrates, it would seem unlikely that accessibility of PMSF to the catalytic serine would be the cause of this weak inhibition, especially when considering the overall similarity between the structures of PMSF and *p*NP-Ac. However, weak inhibition of other serine enzymes by PMSF has been observed, including with *N. gonorrhoeae* Ape1.[16] It is possible that PatB, and these other enzymes, require structural alteration(s) promoted by binding of either ligand and/or protein partner(s) to align appropriately the catalytic triad which would only then render the catalytic Ser nucleophilic for reaction with its natural substrate. If so, presumably PMSF lacks this ability to promote the required conformational change. In this regard, another potential source of activated acetate, acetyl-phosphate does not serve as a cosubstrate for PatB in vitro (unpublished data), and so perhaps it too does not induce the postulated conformational change. Confirmation of such ligand-induced alterations, of course, would require knowledge of the three-dimensional structure of the enzyme, a project that we have been making concerted attempts at accomplishing but with limited success so far.

On the basis of both kinetic and biochemical evidence, we have been able to show for the first time that PG O-acetylation proceeds via a ping-pong bibi catalytic mechanism. This information will be valuable for the identification and development of PG *O*-acetyltransferase inhibitors which could represent potential leads to novel classes of antibiotics that are desperately needed by clinicians combating bacterial infections and diseases. In this regard, the Center for Disease Control and Prevention listed in its 2013 Threat Report drug-resistant *N. gonorrhoeae* as one of three urgent (highest level) threats in the United States. That the β-lactam antibiotics target the Ser nucleophile of the penicillin-binding proteins, enzymes involved in the earlier stages of PG metabolism within the periplasm, together with the discovery of inhibitors with specificity for homoserine transacetylase as possible leads for new antibacterial agents,[33] accentuates the potential of exploiting the Ser nucleophile of PatB as a drugable target.

■ ASSOCIATED CONTENT

Supporting Information

Supplemental figures and methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS

PG, peptidoglycan; MurNAc, *N*-acetylmuramic acid; GlcNAc, *N*-acetylglucosamine; Pat, peptidoglcyan *O*-acetyltransferase; Ape1, *O*-acetylpeptidoglycan esterase 1; Ni^{2+}NTA, Ni^{2+}-nitrilotriacetic acid; *p*NP-Ac, *p*-nitrophenol acetate; CoA, coenzyme A; PMSF, phenylmethylsulfonyl fluoride

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