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Post-synthetic modification of bacterial peptidoglycan using bioorthogonal *N*-acetylcysteamine analogs and peptidoglycan *O*-acetyltransferase B

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Abstract

Bacteria have the natural ability to install protective post-synthetic modifications onto its bacterial peptidoglycan (PG), the coat woven into bacterial cell wall. Peptidoglycan *O*-acetyltransferase B (PatB) catalyzes the *O*-acetylation of PG in Gram (–) bacteria, which aids in bacterial survival, as it prevents autolysins such as lysozyme from cleaving the PG. We explored the mechanistic details of PatB's acetylation function and determined that PatB has substrate specificity for bioorthogonal short *N*-acetyl cysteamine (SNAc) donors. A variety of functionality including azides and alkynes were installed on tri-*N*-acetylglucosamine (NAG)₃, a PG mimic, as well as PG isolated from various Gram (+) and Gram (–) bacterial species. The bioorthogonal modifications protect the isolated PG against lysozyme degradation *in vitro*. We further demonstrate that this post-synthetic modification of PG can be extended to use click chemistry to fluorescently label the mature PG in whole bacterial cells of *Bacillus subtilis*. Modifying PG post-synthetically can aid in the development of antibiotics and immune modulators by expanding the understanding of how PG is processed by lytic enzymes.

Graphical abstract

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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: including experimental methodology, spectral data, PG-isolation protocols, cell-labeling conditions, kinetics data and synthetic procedures.

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Bacterial cell wall, also known as peptidoglycan (PG), is unique to bacteria¹ and is composed of a polymeric network of alternating units of *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM)-pentapeptide carbohydrates that are further cross-linked (Figure 1).² PG is a key component in bacterial cell survival, regulating the osmotic pressure, temperature and pH.³ PG contributes to the bacterial cell shape, and serves as the anchoring point for wall teichoic acids (WTA) and other extracellular proteins.⁴

Bacteria naturally utilize survival mechanisms involving the post-synthetic modification of its PG. Lysozyme, a mammalian antimicrobial enzyme, acts on the PG by cleaving the β -1,4-glycosidic bond between NAG and NAM. *O*-acetylation of PG is found in pathogenic bacterial species including *Staphylococcus aureus* and *Neisseria gonorrhoeae*,⁵ leading to resistance of lysozyme degradation.⁶ The level of *O*-acetylation can be as high as 70% in certain bacterial species.⁷ Despite the prevalence of *O*-acetylation, the source and delivery mechanism of the acetate is debated; it is hypothesized that the natural source of the acetate group is from acetyl-CoA. Since *O*-acetyl groups are not found on precursors in the PG biosynthetic pathway, *O*-acetylation is considered to occur post synthetically on either nascent or mature PG.⁸ Therefore a mechanism through which the acetate derived from acetyl-CoA is translocated from the cytoplasm to the periplasmic space via a transporter has been proposed.⁹

The bacterial enzyme, peptidoglycan *O*-acetyltransferase B (PatB), was first identified as a PG *O*-acetyltransferase in Gram-negative bacteria by Clarke and coworkers.¹⁰ PatB is proposed to function in conjunction with peptidoglycan *O*-acetyltransferase A (PatA), a transmembrane protein (Figure 1a). Once the acetate is in the periplasm, PatB catalyzes the transfer of an acetyl group onto the 6-hydroxyl position of NAM (Figure 1a).¹¹ The Clarke group has eloquently demonstrated that PatB accepts *para*-nitrophenol-acetate (*p*NP-Ac) (**1**, Figure 2a), to label small molecule PG mimics. In addition, it was demonstrated that PatB hydrolyzes propionyl CoA.¹² Intrigued by PatB's promiscuity, we sought to install bioorthogonal functionality onto the PG, which could help to illuminate the mechanism and extent of modification of *O*-acetylation.

The substrate specificity of PatB's activity was explored with wild type PatB expressed with Glutathione *S*-transferase (GST) and His₆ tags on the *N*-terminal and *C*-terminal, respectively to improve stability and purification (Figure S1).¹³ PatB's esterase activity was measured using an established *p*NP release assay.¹⁴ The rate of *p*NP release from **1** increased in the presence of PatB (Figure S1c). We found that PatB was able to acetylate a trisaccharide, tri-*N*-acetyl-glucosamine (NAG)₃ (Figure 2b) (Table S1). This acceptor specificity is in agreement with previous work where it was demonstrated that PatB requires acceptors that are trisaccharides or longer and the acetylation of the amino group at the C2 position is crucial for PatB's activity.¹²

We next determined if other donors could be utilized. A variety of acetyl donors, containing alkyne, azido, and phenyl functionalities, were synthesized (**2–4**, Figure 2a) and assayed for the ability to acetylate the model glycan acceptor (NAG)₃. High resolution mass spectrometry confirmed that PatB was able to use **2–4** to modify (NAG)₃ (Table S1). Kinetic parameters for acetyl transferase were measured by *p*NP release (Table S2). The *K_m* values

are in the 0.7–1.5 μM range and overall $k_{\text{cat}}/K_{\text{m}}$ values were approximately forty times lower for the full length PatB than previously reported for a truncated SUMO tagged PatB.¹² In order to efficiently install the bioorthogonal functionality using full length PatB, modified acetyl donors were explored.

The proposed natural source of the acetate functional group utilized by PatB is acetyl-CoA (**5**, Figure 2c).¹¹ PatB modified (NAG)₃ using **5** (Table S1). It was hypothesized that smaller variants of **5** could be used as donors, as a variety of CoA ester-utilizing enzymes are reported to accept that smaller variants of **5** could be used as donors, as a variety of CoA ester-utilizing enzymes are reported to accept truncated CoA thioesters known as *N*-acetylcysteamine-thioesters (SNAc) (Figure 1b).¹⁵ Moreover, the SNAc-thioesters would be less susceptible to non-enzymatic hydrolysis as compared to the *p*NP donors. In 2005, Pohl and coworkers used SNAc analogs to prepare labeled sugar nucleotides with bioorthogonal functionality,¹⁶ noting that SNAcs are advantageous compared to their CoA derivatives due to their synthetic simplicity.¹⁷ To assess the utility of SNAc compounds as PatB donors, *S*-(2-acetamidoethyl)-ethanethioate (SNAc, **6**)^{17a}, a novel azido SNAc derivative *S*-(2-acetamidoethyl)-2-azidoethanethioate (SNAz, **7**) along with an alkynyl form, *S*-(2-acetamidoethyl)-2-alkynylethanethioate (SNAk, **8**)^{17b} were synthesized (Figure 2c). In addition, SNAc donors with extended core structures (**9** and **10**) as well as a larger, fluorescent acetyl moiety (SNBodipy, **11**) were synthesized to probe the substrate specificity of PatB. PatB was able to modify (NAG)₃ using **6–10** as confirmed by HRLC/MS (Table S1). PatB was unable to transfer donor **11** onto the (NAG)₃ sugar. Low solubility of **9–11** may attribute to the lower transfer activity as observed by MS analysis; thus the smaller and more synthetically accessible SNAc derivatives **6–8** were chosen as optimal substrates for PatB. With the (NAG)₃ alkyne modification successfully installed from **8**, Alexa- Fluor488 was successfully attached via click chemistry¹⁸ (Copper catalyzed azide-alkyne-cycloaddition (CuAAC)) (Figure S3).

To measure the catalytic efficiency of PatB with **6–8** in the presence of (NAG)₃, a colorimetric assay using Ellman's reagent¹⁹ was employed. The $k_{\text{cat}}/K_{\text{m}}$ values for the SNAc donors were low at pH 6.5 (Table 1). We reasoned that pH of the reaction could be important, as the natural environment (the periplasm of *N. gonorrhoeae*) in which PatB resides is slightly basic.²⁰ At pH 8.5, the catalytic efficiencies of PatB are 10–20X higher than those observed at pH 6.5 (Table 1).

To evaluate PatB's acetyltransferase activity on larger, biologically relevant glycans, we took advantage of its natural ability to prevent the degradation of PG *in situ*. PG was isolated from a bacillus strain with the *O*-acetyltransferase (Oat) gene knocked-out (*B. sub. Oat*). The peptidoglycan from this mutant strain has been shown to be devoid of any natural acetylation at the C6-OH position of NAM.²¹ The insoluble PG was subjected to lysozyme degradation in the presence and absence of PatB with **1, 6–8**; the optical density was measured over time to monitor the increase or decrease of PG degradation process.²² The optical density of unmodified PG decreased over time with lysozyme treatment, indicating that the PG was being cut into soluble fragments from lysozyme treatment (Figure 3a, Figure S1d). When the PG was pre-treated with PatB and **1, 6, 7** or **8**, then the polymer was protected against lysozyme degradation (Figure 3a, Figure S1d). Encouraged by these

results, PG was isolated from other bacterial species *Escherichia coli*, *Vibrio parahaemolyticus*, and *Pseudomonas putida* (Supplemental Methods); as these species have not been shown to *O*-acetylate their PG.^{7,23} PatB conferred lysozyme resistance to the isolated PG from all of the bacterial strains (Figure 3b–d). As expected, some degradation of the acetylated PG was observed, as PatB may not be able to modify every NAM residue in the PG. The data demonstrate that PatB is capable of modifying small and large fragments of PG with bioorthogonal functionality.

Intrigued by the ability of the bioorthogonal SNAc donors to modify isolated PG, *in vivo* labeling of Gram-positive bacterial cells using PatB was pursued. Inspiration to tag the PG polymer was based on previous strategies used to label either the peptide stems²⁴ or carbohydrate core²⁵ of PG. While these methods rely on metabolic incorporation of unnatural PG building blocks, PatB assisted labeling would be a complimentary method that relies on Nature's ability to post-synthetically modify PG to install bioorthogonal functionality on the carbohydrate portion of PG, more specifically the 6-OH of NAM, which to date remains an unexplored PG labeling target. Similar enzymatic approaches have been employed by the Spiegel lab to use sortase A (SrtA) as a tool for incorporating molecular handles onto the cell wall of *S. aureus*^{26a} and by Liu and Tam to label *E. coli* cell surfaces through a butelase-mediated ligation method to a transmembrane protein.^{26b}

B. subtilis Oat and wild-type cells were treated with PatB and donor **6** or **8**. CuAAC was implemented to label the cells with Alexa Fluor 488-Az and subsequently imaged via Structured Illumination Microscopy (SIM).²⁷ A time course determined that optimal labeling was achieved at 240 min (Figure S4). In both *B. subtilis* Oat and wild-type strains, cells displayed higher fluorescence signal intensity when treated with PatB and **8** as compared to treatment with **8** alone or PatB with **6**, both of which showed low background auto-fluorescence (Figure 4, Figure S5). These results indicate that PatB is necessary for efficient labeling of bacterial cells using SNAc derivatives. Fluorescence in the wild-type *B. subtilis* cells treated with only **8** could be attributed to OatA's presence as the fluorescence is absent when Oat cells are treated with only **8**. To verify that labeling is specific to PG, cells were stained with a tetramethylrhodamine-conjugate of wheat germ agglutinin (WGA), which binds to NAG.²⁸ In addition, cultures of *B. subtilis* Oat were treated with lysozyme (Fig. S6),²¹ and a protective effect for cultures grown in the presence of PatB/**6** was observed, supporting PG acetylation. These verification approaches were chosen over a MS-based assay as the acetylated PG is resistant to lysozyme degradation²⁹ making isolation of detectable fragments extremely difficult. Moreover, the fragile acetyl modifications are lost during the harsh PG isolation.³⁰ The co-localization of Alexa-Fluor488 and tetramethylrhodamine-WGA in the samples treated with PatB and **8** suggest that the labeling was specific to the PG (Figure 4). The Oat cells have a higher fluorescence signal as compared to the wild-type, which can be attributed to more sites available for PatB modification in Oat cells. Interestingly, the fluorescence signal appears to localize around the septal-division ring.³¹ Nascent PG is proposed to form at this ring during cell division,³² thus it is plausible that PatB is more likely to target the unmodified PG found in this area. We propose that the labeling methodology presented here was successful and specific in labeling PG from *B. subtilis* cells.

The SNAc derivatives employed here shed light on the mechanism of action of PatA, the pro-posed acetyl carrier. It is currently unknown how PatA translocates the acetate moiety of acetyl-CoA (Figure 1a). PatB accepts SNAc donors, which contain a thioester bond, suggesting that the acetate group of acetyl-CoA is possibly linked to a cysteine residue of PatA.

To conclude, PatB has relaxed substrate specificity for both *p*NP and SNAc donors, allowing for the covalent attachment of bioorthogonal functionality directly onto a small molecule PG fragment mimic, isolated PG from *B. subtilis*, *E. coli*, *V. parahaemolyticus*, and *P. putida*, and intact *B. subtilis* cells post-synthetically. The modifications protected the PG against lethal lysozyme degradation mirroring Nature's ability to post-synthetically modify PG. In addition, wild-type PatB has an optimal catalytic efficiency at basic pH, which reflects its natural environment. PatB's utility to incorporate fluorescent tags via click chemistry on the PG both *in vitro* and in whole cells allowed for fluorescent visualization of bacterial cells. PatB proves to be a useful tool in understanding the acetylation modification of PG and its resistance mechanisms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

A) An acetyl functional group (red) is translocated across the plasmic membrane by PatA, where it is subsequently transferred onto the sixth hydroxyl position of NAM by PatB. B) Unnatural SNAC donors employed to install bioorthogonal functional groups (blue) onto the peptidoglycan by PatB.

Figure 2.

PatB Donors and Acceptors: A) Donors, which retained the *p*NP moiety, were synthesized and tested with PatB. B) Monosaccharide and trisaccharide acceptors tested with PatB. C) The natural substrate, acetyl-CoA, *N*-acetyl cysteamine derivatives, which contained bioorthogonal handles, were synthesized and tested to probe for PatB promiscuity.

Figure 3.

Lysozyme Degradation Assay: Isolated peptidoglycan from *B. subtilis* Oat (A), *E. coli* (B), *P. putida* (C) and *V. parahaemolyticus* (D) were protected against lysozyme after modification by PatB and **6** (purple), **7** (black) or **8** (orange) as compared to the unmodified peptidoglycan (green). Also shown are blank (blue) and PG without lysozyme (red). Data shown are averages of three independent reactions. Error bars represent 1 standard deviation of the mean.

Figure 4.

SIM and corresponding DIC images of *B. subtilis* (ΔOat and wild-type) cells labeled with Alexa-Fluor488 (green, top row) post modification by PatB and **8**, PatB and **6**, or **8** alone. The cell wall was also stained with a tetramethylrhodamine-WGA-conjugate (red, second row) to show colocalization of the fluorophores (yellow, third row). Images are representative of three biological replicates (Fig. S5 for large fields). Scale-bar = 2 μm.

Table 1

Kinetic Data for acetyl-CoA and SNAc donors^a Standard deviation of the analysis was from the average of three independent kinetic experiments.

Entry	Donor	Acetyltransferase Activity	
		pH = 6.5	pH = 8.5
1	5	$k_{\text{cat}} = 0.00544 \pm 0.00026$	$k_{\text{cat}} = 0.422 \pm 0.018$
		$K_{\text{m}} = 0.204 \pm 0.012$	$K_{\text{m}} = 1.62 \pm 0.17$
		$k_{\text{cat}}/K_{\text{m}} = 26.69 \pm 3.74$	$k_{\text{cat}}/K_{\text{m}} = 260.2 \pm 20.1$
2	6	$k_{\text{cat}} = 0.0179 \pm 0.0025$	$k_{\text{cat}} = 1.62 \pm 0.06$
		$K_{\text{m}} = 0.327 \pm 0.030$	$K_{\text{m}} = 1.47 \pm 0.23$
		$k_{\text{cat}}/K_{\text{m}} = 54.82 \pm 7.16$	$k_{\text{cat}}/K_{\text{m}} = 1,103 \pm 91$
3	7	$k_{\text{cat}} = 0.0370 \pm 0.0033$	$k_{\text{cat}} = 0.617 \pm 0.071$
		$K_{\text{m}} = 0.661 \pm 0.084$	$K_{\text{m}} = 1.12 \pm 0.14$
		$k_{\text{cat}}/K_{\text{m}} = 55.99 \pm 6.58$	$k_{\text{cat}}/K_{\text{m}} = 527.1 \pm 60.6$
4	8	$k_{\text{cat}} = 0.0288 \pm 0.0048$	$k_{\text{cat}} = 1.85 \pm 0.056$
		$K_{\text{m}} = 0.531 \pm 0.053$	$K_{\text{m}} = 1.69 \pm 0.22$
		$k_{\text{cat}}/K_{\text{m}} = 54.21 \pm 6.19$	$k_{\text{cat}}/K_{\text{m}} = 1,094 \pm 93$

^a k_{cat} [sec^{-1}], K_{m} [mM], $k_{\text{cat}}/K_{\text{m}}$ [$\text{M}^{-1}\text{sec}^{-1}$]