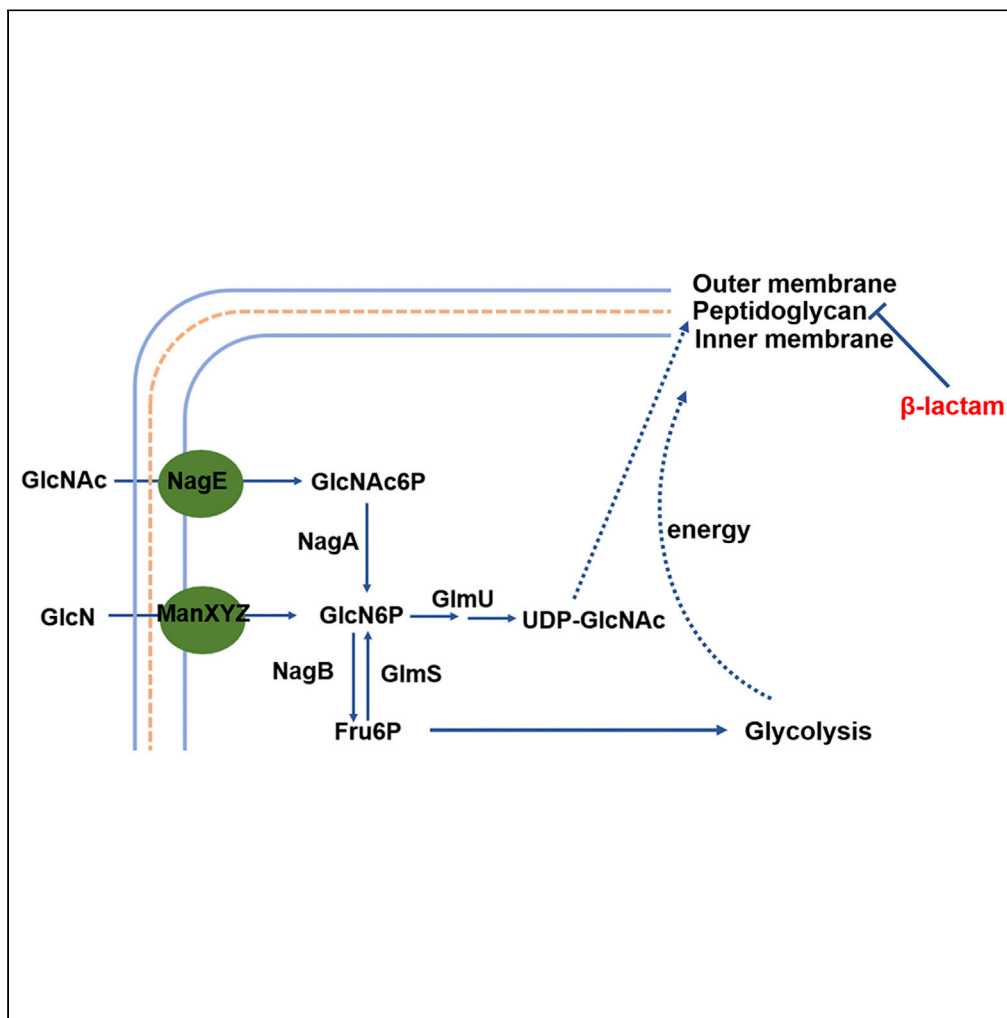


Article

N-Acetyl-D-Glucosamine Acts as Adjuvant that Re-sensitizes Starvation-Induced Antibiotic-Tolerant Population of *E. Coli* to β -Lactam



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HIGHLIGHTS

Exogenous GlcNAc renders antibiotic-tolerant *E. coli* susceptible to β -lactams

Exogenous GlcNAc re-activates peptidoglycan synthesis under starvation

GlcNAc-elicited peptidoglycan synthesis also involves activation of glycolysis

GlcNAc re-sensitization effect is not associated with cAMP and ROS production

Wang et al., iScience 23,
101740
November 20, 2020 © 2020
The Authors.
[https://doi.org/10.1016/
j.isci.2020.101740](https://doi.org/10.1016/j.isci.2020.101740)

Article

N-Acetyl-D-Glucosamine Acts as Adjuvant that Re-Sensitizes Starvation-Induced Antibiotic-Tolerant Population of *E. Coli* to β -Lactam

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SUMMARY

Bacterial tolerance to antibiotics causes reduction in efficacy in antimicrobial treatment of chronic and recurrent infections. Nutrient availability is one major factor that determines the degree of phenotypic antibiotic tolerance. In an attempt to test if specific nutrients can reverse phenotypic tolerance, we identified N-acetyl-D-glucosamine (GlcNAc) as a potent tolerance-suppressing agent and showed that it could strongly re-sensitize a tolerant population of *E. coli* to ampicillin. Such re-sensitization effect was attributable to two physiology-modulating effects of GlcNAc. First, uptake of GlcNAc by the tolerant population triggers formation of the peptidoglycan precursor UDP-N-acetyl-D-glucosamine (UDP-GlcNAc) and subsequently re-activates the peptidoglycan biosynthesis process, rendering the organism susceptible to β -lactam antibiotics. Second, activation of glycolysis by-products of GlcNAc catabolism drives the re-sensitization process. Our findings imply that GlcNAc may serve as a non-toxic β -lactam adjuvant that enhances the efficacy of treatment of otherwise hard-to-treat bacterial infections due to phenotypic antibiotic tolerance.

INTRODUCTION

Current antimicrobial regimens are often ineffective in completely eradicating the infecting agents in treatment of bacterial infections, predisposing occurrence of relapsed and chronic infections. Antibiotic-tolerant sub-populations of non-resistant strains are recalcitrant to the bactericidal effects of even a high dose of antimicrobials, and are often responsible for failure of antimicrobial therapy, for example 26.6% women experienced recurrence of urinary tract infection of *E. coli* within 6 months of the initial infection (Fisher et al., 2017; Foxman, 1990; Russo et al., 1995). Antibiotic tolerance is a reversible phenotype initiated stochastically or by environmental stresses and is characterized by a slow or non-growing state, which renders antimicrobial action ineffective; yet a tolerant bacterial sub-population can rapidly resume active growth when environmental conditions become favorable (Bernier et al., 2013; de Steenwinkel et al., 2010; Horne and Tomasz, 1977; Yu et al., 2019).

Based on the current understanding of cellular mechanisms that underlie tolerance formation, several strategies have been proposed to eradicate antibiotic-tolerant bacterial cells. The first approach involves direct killing of metabolically inactive tolerant cell. Two synthetic retinoid compounds, CD437 and CD1530, were found to exhibit killing effects on *S. aureus* (methicillin-resistant *S. aureus* [MRSA])-tolerant cells that are unresponsive to methicillin by disrupting the membrane bilayers (Kim et al., 2018). Yet these compounds, which were previously used as anti-tumor agents, are highly toxic and hence unsuitable for treatment of patients who suffer from serious bacterial infections (Han et al., 2016; Valli et al., 2008). Another compound, ADEP4, which renders Clp protease ATP independent, was found to promote protein digestion and kill tolerant *M. tuberculosis* and *S. aureus* organisms (Conlon et al., 2013). The second approach aims to resuscitate tolerant cells or re-sensitize them to antibiotics. *Cis*-2-decenoic acid, a fatty acid signaling molecule, was found to revert the dormant cells to a metabolically active state, which is susceptible to antimicrobial agents (Marques et al., 2014). Metabolites such as glucose, fructose, pyruvate, and mannitol were also reported to sensitize tolerant cells to gentamicin by promoting the generation of a proton motive force (PMF), which is required for aminoglycoside uptake (Allison et al., 2011; Barraud et al., 2013; Meylan et al., 2017). The combined usage of several conventional antibiotics also exerts synergistic bactericidal effect on tolerant cells' effect. For example, daptomycin boosts the efficacy of

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<https://doi.org/10.1016/j.isci.2020.101740>



eradication of *Borrelia burgdorferi*-tolerant cells by β -lactams (Feng et al., 2015). The third approach is to interfere with the tolerance formation process. It was reported that nitric oxide perturbs tolerance formation and renders bacterial cells susceptible to ampicillin or ofloxacin by inhibiting respiration (Orman and Brynildsen, 2016). Benzamide-benzimidazole-type compounds enable tolerant cells to be killed by meropenem, ciprofloxacin, and amikacin by inhibiting quorum sensing (Starkey et al., 2014). PKUMDL-LTQ series compounds, which are inhibitors of the toxin HipA, were reported to exhibit an effect of reducing the level of bacterial tolerance to ampicillin and kanamycin (Li et al., 2016). Likewise, phage-encoded expression of LexA3 inhibits SOS response-mediated DNA repair, rendering both planktonic and biofilm-associated tolerant cells susceptible to quinolones, β -lactams, and aminoglycosides (Lu and Collins, 2009).

Clinical application of the aforementioned methods for eradication of bacterial tolerant sub-population has been limited by drawbacks of each of these methods. For example, compounds that mediate killing of tolerant cells are often toxic to mammalian cells or are not suitable for clinical application (Kim et al., 2018). In this work, we screened for nutrients that can suppress starvation-induced tolerance response, so as to develop a non-toxic and clinically feasible anti-tolerance therapeutic approach. We found that *N*-acetyl-D-glucosamine (GlcNAc) and D-glucosamine (GlcN) could strongly re-sensitize bacterial tolerant sub-population that emerged during nutrient starvation stress to β -lactams. GlcN·HCl was previously reported to exhibit a strong antimicrobial effect on *Staphylococcus saprophyticus* and *Micrococcus luteus* (Malik et al., 2013). GlcNAc is also known to induce death of tolerant cells in *Candida albicans* by triggering reactive oxygen species (ROS) (Du et al., 2015). GlcNAc is a major component of the bacterial cell wall, a matrix composed of alternating GlcNAc and *N*-acetylmuramic acid (MurNAc) molecules cross-linked by peptide bridges and are the target of β -lactams. Both GlcNAc and GlcN are converted to glucosamine-6-phosphate (GlcN6P) upon transportation into the cytosol (Mukhija and Erni, 1996; Souza et al., 1997). GlcN6P could enter the hexosamine pathway and become converted to UDP-*N*-acetyl-D-glucosamine (UDP-GlcNAc), the precursor for synthesis of peptidoglycan; it may also be catabolized to Fru6P, a substrate of glycolysis (Midelfort and Rose, 1977; Rodriguez-Diaz et al., 2012).

Despite the previous findings, the mechanisms by which GlcNAc or GlcN inhibits bacterial antibiotic tolerance remain poorly defined. We hypothesize that GlcNAc re-sensitizes tolerant bacterial cells to β -lactams through the following mechanisms. First, GlcNAc triggers activation of peptidoglycan synthesis by generation of UDP-GlcNAc, so that the target of β -lactams becomes functionally active. Hurdle et al. proposed that the bacterial membrane bilayer and membrane proteins, which are integral to membrane function, were ideal targets of potential tolerance-inhibitory agents, as even tolerant cells need to maintain an integrated membrane and cell wall to remain viable (Hurdle et al., 2011). Bacteriophage-derived lysine CF-301 is known to exhibit pronounced MRSA killing effect by causing cell lysis by eliciting peptidoglycan degradation (Schuch et al., 2014). GlcNAc does not cause peptidoglycan degradation; instead it activates the peptidoglycan synthesis processes in tolerant cells, thereby re-sensitizing them to β -lactams. Second, GlcNAc catabolism produces Fru6P, which in turn promotes onset of glycolysis, revives oxidative phosphorylation and ATP production, and subsequently reverts the dormant cells to at least a partially metabolically active and β -lactam-sensitive state. Based on findings in this work, we believe that GlcNAc-mediated sensitization of tolerant cells to β -lactam is a highly effective and feasible tolerant cell eradication approach.

RESULTS

Exogenous GlcNAc Drastically Enhances Susceptibility of Starvation-Induced Antibiotic-Tolerant Population of *E. Coli* to β -Lactam Both *In Vitro* and *In Vivo*

Bacteria become highly tolerant to multiple antibiotics upon complete deprivation of all essential nutrients (Fung et al., 2010; Nguyen et al., 2011). We hypothesize that such starvation-mediated tolerance phenotype is not solely due to shutdown of metabolic activities but also involved in the activation of cellular mechanisms that enhance the capability of bacteria to survive environmental stresses, and that starvation-induced protective mechanisms can be suppressed by supplementation of specific nutrients. To test these hypotheses, we screened for compounds that could suppress phenotypic tolerance by re-sensitizing the bacterial sub-population to ampicillin. Among a total of 95 carbon sources in the Phenotype MicroArrays (PM plates, Biolog) tested (Figure S1), we identified GlcNAc as the compound that exhibited the highest potential in re-sensitizing the tolerant sub-population to ampicillin. In the presence of this compound, ampicillin was able to eradicate a significant proportion of tolerant sub-population, so that only a small number of bacteria in such population were able to survive and re-grow in the screening assay (Figure S1). GlcNAc was non-toxic,

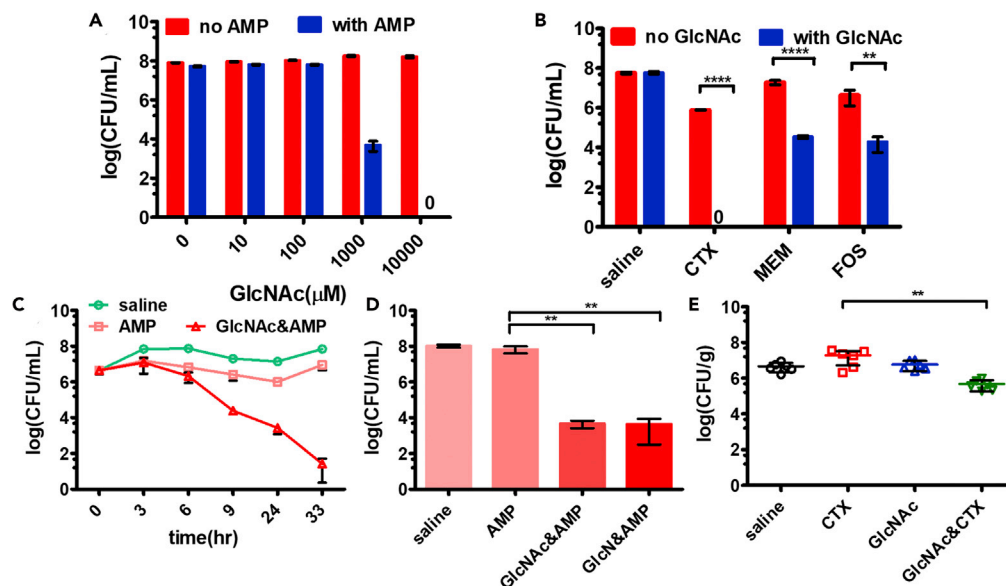


Figure 1. Effect of Supplementation of Exogenous GlcNAc on Susceptibility of Starvation-Induced Tolerant *E coli* Sub-population to β -Lactams both *In Vitro* and *In Vivo*

(A) Starved *E. coli* were incubated with increasing concentrations of GlcNAc for 24 h in the presence and absence of ampicillin (AMP, 100 μ g/mL).
 (B) Starved *E. coli* were incubated with different β -lactams for 24 h in the presence or absence of GlcNAc (1 mM); cells re-suspended in saline were included as negative control. CTX, cefotaxime (100 μ g/mL); MEM, meropenem (40 μ g/mL); FOS, fosfomycin (40 μ g/mL).
 (C) Starved *E. coli* were incubated with ampicillin for up to 33 h in the presence or absence of GlcNAc (1 mM); cells re-suspended in saline were included as negative control.
 (D) Stationary phase *E. coli* cells were re-suspended in saline and then incubated with ampicillin for 24 h in the presence of GlcNAc (1 mM) or GlcN (1 mM); cells re-suspended in saline were included as negative control.
 (E) *E. coli* mice deep-seeded thigh infection model. 1×10^7 cells were injected to the two thighs of each test mouse. At 24 h post-infection, the mice were subjected to the indicated dosage of antibacterial treatment (intraperitoneally) every 12 h for 72 h. Mice were euthanized and the thighs were aseptically excised, homogenized in saline, and the number of bacteria that survived determined (expressed in CFU). Results are displayed as mean \pm SD, and the degree of significance was determined by unpaired, two-sided Student's test (ns, no significance; ** $p < 0.01$; **** $p < 0.0001$). Results of animal studies were analyzed by one-way ANOVA and post-hoc Tukey test (** $p < 0.01$). Three biological repeats were carried out.

See also [Figures S1 and S2](#).

as its presence did not affect bacterial growth in LB broth (Figure S2A). We also determined if GlcNAc could support re-growth of a starvation-induced antibiotic-tolerant population. In the presence of GlcNAc, the size of the bacterial population that had been subjected to starvation for 24 h by re-suspending in saline increased only about 2-fold in a 24-h period ($\sim 1.2 \times 10^8$ CFU/mL), when compared with the negative control in which GlcNAc was not supplemented ($\sim 5.5 \times 10^7$ CFU/mL). Considering the large original cell population, the effect of GlcNAc on bacterial proliferation is negligible (Figure S2B). We then further assessed the potential of GlcNAc to re-sensitize a starvation-induced tolerant population to ampicillin. Treatment with 100 μ g/mL ampicillin for 24 h was only able to eradicate a very small proportion of the test population, with $\sim 5 \times 10^7$ CFU/mL of the original population of $\sim 10^8$ CFU/mL being able to survive the treatment, indicating that a large tolerant sub-population formed during nutrient starvation. In the presence of GlcNAc, however, the survival fitness of this tolerant sub-population was found to decrease drastically upon ampicillin treatment. When compared with the sample treated with ampicillin alone, the survival rate of the tolerant sub-population decreased ~ 4 logs upon treatment with ampicillin in the presence of 1 mM GlcNAc (Figure 1A). With an increasing GlcNAc concentration or increasing treatment time, tolerant cells could be almost completely eradicated (Figures 1A and 1C). To further confirm the effect of GlcNAc, we also tested whether GlcN, which can be assimilated by bacteria in a manner similar to that of GlcNAc, could re-sensitize tolerant sub-population. The re-sensitization effect of GlcN was found to be similar to that of GlcNAc (Figure S2C).

Apart from ampicillin, GlcNAc was also found to re-sensitize starvation-induced tolerant cells to other β -lactams and fosfomycin, which target peptidoglycan synthesis. β -Lactam antibiotics target and inhibit transpeptidase, commonly known as penicillin-binding proteins, which mediate the final assembly of peptidoglycan by catalyzing cross-linkage between the neighboring stem peptides via formation of pentaglycine bridges. Fosfomycin prevents the formation of UDP-GlcNAc-3-O-enolpyruvate from UDP-GlcNAc, the first step in peptidoglycan biosynthesis (Kahan et al., 1974). In the presence of GlcNAc, the susceptibility of starvation-induced tolerant cells to cefotaxime sharply increased (Figure 1B). Cell survival in the presence of meropenem or fosfomycin plus GlcNAc was two to three orders of magnitude lower than that recorded upon treatment with meropenem or fosfomycin alone (Figure 1B). Apart from their re-sensitization effect on starvation-induced antibiotic-tolerant sub-population, GlcNAc and GlcN were also able to re-sensitize stationary phase cells to ampicillin, with both being able to cause 4 log reduction in the size of the antibiotic-tolerant stationary phase population when used in combination with ampicillin (Figure 1D). The re-sensitization effect of GlcNAc was not only observable in stationary phase cells re-suspended in saline, and also in those re-suspended in spent media of stationary phase culture. In each case, a 6 log reduction in the size of the bacterial population was recorded following treatment with GlcNAc and ampicillin (Figure S2D). We also tested the effect of GlcNAc on a clinically isolated *E. coli* strain that is resistant to meropenem and found that survival of this strain upon encountering nutrient starvation stress also decreased ~ 4 log when treated with GlcNAc and meropenem, indicating that β -lactam-resistant strains may also be killed by this approach when they enter the starvation-induced tolerance mode (Figure S2E).

The efficacy of the GlcNAc and β -lactam combination to eradicate bacterial tolerant sub-population was further investigated in a mouse infection model. A deep-seeded thigh tolerance model using *E. coli* BW25113 was established, with cefotaxime being used as the test agent; the results showed that the size of the surviving bacterial population recorded after treatment with the GlcNAc and cefotaxime combination was 4.8×10^5 CFU/g, which was significantly lower than that recorded after treatment with cefotaxime alone (1.9×10^7 CFU/g) (Figure 1E). Interestingly, GlcNAc did not enhance the susceptibility of the tolerant sub-population to aminoglycosides as cell survival did not change when GlcNAc was included in treatment with gentamicin, kanamycin, or streptomycin, suggesting that the underlying mechanism of GlcNAc-mediated sensitization was different from that mediated by glucose and fructose, which rendered tolerant cells susceptible to aminoglycosides (Figure S2F). Also, phenotypic tolerance was not altered when GlcNAc was used in combination with ciprofloxacin or rifampin (Figure S2F); this observation was consistent with a former report that showed that supplementation of metabolic intermediates in the glycolysis pathway did not cause tolerant cells to become susceptible to quinolones (Allison et al., 2011).

Morphology of Ampicillin-Treated Cells in the Presence of GlcNAc or GlcN

Scanning electron microscopic (SEM) study was performed on *E. coli* strain that had been treated with GlcNAc or GlcN in the presence or absence of ampicillin to examine the morphological changes in tolerant cells exposed to these compounds. Compared with the untreated cells, which had integrated and smooth membrane and exhibited homogeneous plasma distribution, cells treated with the GlcNAc and ampicillin combination showed signs of extensive membrane damage including cell lysis, release of cellular contents, and lack of cellular features (Figure 2). The morphology of bacteria subjected to treatment with GlcNAc alone was identical to that of the untreated control, with integrated and smooth membrane and homogeneous plasma distribution, confirming that GlcNAc itself did not exert any cellular damages. The cell structure and membrane integrity were not altered if only ampicillin was involved in treatment, and the cellular contents were homogeneously distributed without any sign of leakage, indicating that the bacterial cells were tolerant to ampicillin under starvation stress (Figure 2). Likewise, cell lysis and leakage of cellular content were observed upon treatment with the GlcN and ampicillin combination, but not GlcN alone (Figure S3). The SEM observations are consistent with the results of phenotypic assays, which showed that GlcNAc or GlcN could effectively re-sensitize tolerant cells to β -lactams.

GlcNAc Stimulates Peptidoglycan Synthesis

We next investigated the molecular basis of the re-sensitization effect of GlcNAc. Transportation of GlcNAc into the bacterial cell is accompanied by phosphorylation and deacetylation, becoming glucosamine 6-phosphate (GlcNAc6P) and then GlcN6P (NagA; EC 3.5.1.25). Likewise, glucosamine (GlcN) is also converted to GlcN6P upon entry into the bacterial cell (Postma et al., 1993), which then enters the GlcNAc metabolic pathway (Figure 7). In this work, we chose GlcNAc as a test compound and investigated the mechanism that underlies the strong GlcNAc re-sensitization effect. First, we hypothesized that the re-sensitization effect on bacterial

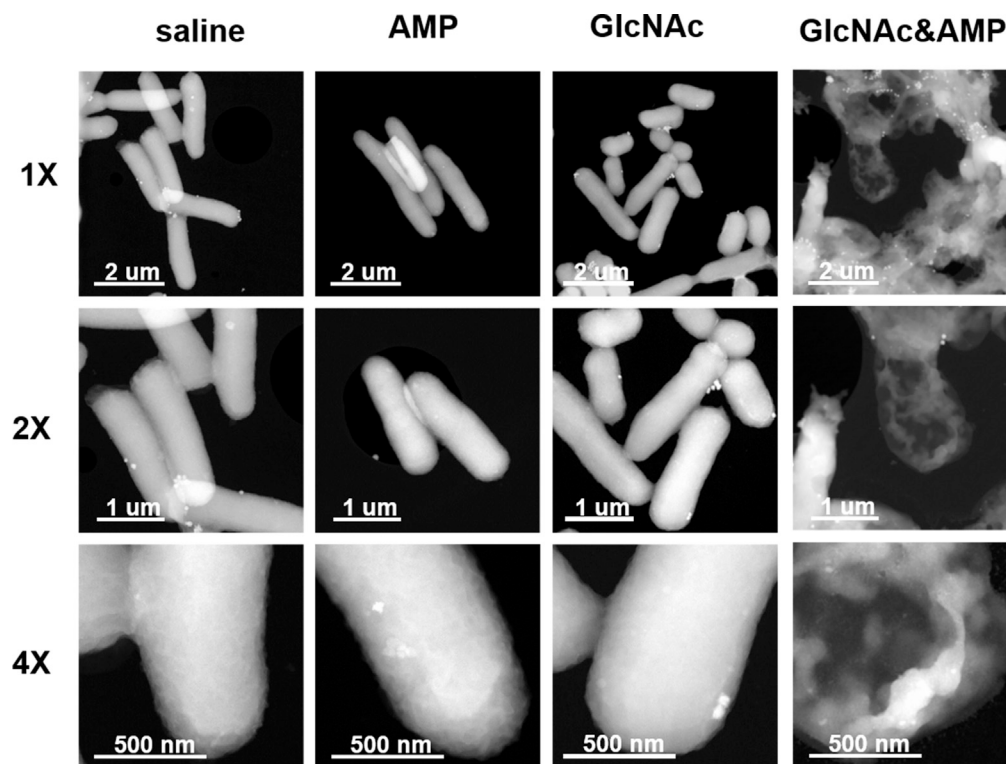


Figure 2. SEM Images of *E. Coli* Cells Treated with Ampicillin, GlcNAc, or the Combination of Both

Starved cells were incubated with ampicillin only (100 $\mu\text{g}/\text{mL}$), GlcNAc only (1 mM), or GlcNAc plus ampicillin (saline as negative control) for 24 h before taking the SEM image. See also [Figure S3](#)

antibiotic tolerance exhibited by GlcNAc was due to its ability to activate the GlcNAc metabolic pathway; we investigated if knockout of the *nagA* gene (ΔnagA), which encodes GlcNAc6P deacetylase and plays a role in converting GlcNAc6P to GlcN6P, could diminish the strength of tolerance re-sensitization. Our data showed that the cell survival rate of the ΔnagA strain upon treatment with ampicillin did not alter when supplemented with GlcNAc, indicating that activation of the GlcNAc metabolic pathway is necessary to convert tolerant cells back to the ampicillin-susceptible mode ([Figure 3A](#)).

In the GlcNAc metabolic pathway, GlcNAc is eventually converted to UDP-GlcNAc, which then enters the peptidoglycan biosynthesis pathway. It is widely accepted that a switch to physiological dormancy underlies formation of bacterial antibiotic tolerance, as the targets of antibiotics are inactive in the dormant state ([Harms et al., 2016](#); [Lewis, 2007](#)). If a specific antimicrobial target can be converted to at least a partially active state by metabolite stimulation, tolerant cells should become sensitive to antibiotics. To examine whether supplementation of GlcNAc can activate peptidoglycan synthesis in tolerant cells, we quantified the amount of intracellular UDP-GlcNAc after treating the tolerant cells with GlcNAc for 24 h. The concentration of UDP-GlcNAc in cells after starvation for 24 h was found to be 0.06 μM , but increased ~ 80 -fold upon treatment with GlcNAc (4.95 μM) ([Figures 3B, 3C, and S4](#)).

The sharply increased amount of UDP-GlcNAc detectable in tolerant cells upon supplementation with GlcNAc suggests that dormant tolerant cells had resumed GlcNAc metabolism and peptidoglycan biosynthesis. To further confirm that peptidoglycan was being synthesized in tolerant cells, we tested whether the fluorescent D-amino acid HADA (7-hydroxycoumarin-3-carboxylic acid-3-amino-D-alanine), which specifically binds to and stains newly synthesized peptidoglycan, could stain the cell wall of tolerant cells in the presence of GlcNAc ([Kuru et al., 2012](#)). Our data showed that none of the tolerant cells under starvation could be stained by HADA, indicating that peptidoglycan biosynthesis has grounded to a halt. However, most of the tolerant cells tested could be stained by HADA 24 h after supplementation with GlcNAc, indicating that peptidoglycan biosynthesis had resumed when GlcNAc was available ([Figure 4A](#)) and that active peptidoglycan biosynthesis elicited by GlcNAc renders tolerant cells susceptible to β -lactams.

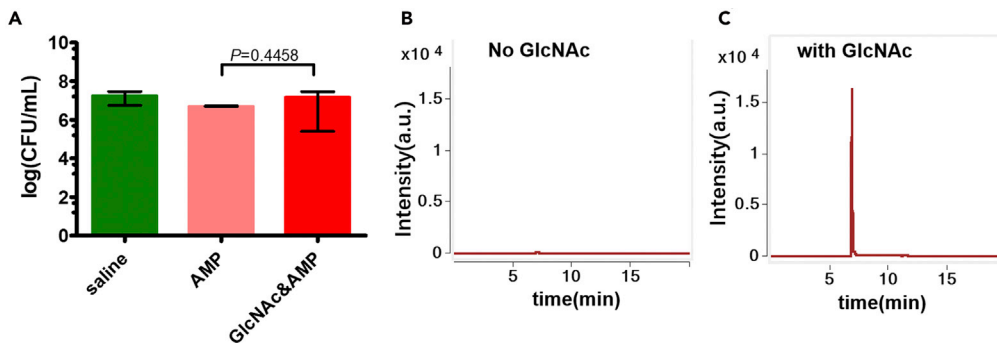


Figure 3. Exogenous GlcNAc Stimulates Peptidoglycan Synthesis

(A–C) (A) Population size of starved $\Delta nagA$ mutant strains incubated with ampicillin for 24 h in the presence or absence of GlcNAc (1 mM); cells re-suspended in saline were included as negative control. The intracellular amount of UDP-GlcNAc detectable in the absence (B) or presence (C) of GlcNAc was measured. Chromatograms of the liquid chromatographic-mass spectrometric (LC/MS) analysis of UDP-GlcNAc in the samples are presented (m/z of 606.2 to 385 collision energy 25V and retention time of 7 min in the HPLC column). Survival rate of tolerant cells is displayed as mean \pm SD; the degree of significance in difference between the test and control samples was determined by unpaired, two-sided Student's test. Three biological repeats were carried out.

See also [Figure S4](#)

Exogenous GlcNAc Promotes Glycolysis

Apart from being a precursor of peptidoglycan biosynthesis, GlcNAc could also be catabolized to Fru6P by the enzyme deaminase, which is encoded by the *nagB* gene (Alvarez-Anorve et al., 2009) (Figure 7). Fru6P is a substrate of glycolysis. Fru6P was also found to exhibit ampicillin re-sensitization effect in the PM assay in this work (Figure S1). To determine whether supplementation of GlcNAc caused an increase in the amount of Fru6P and subsequently activated glycolysis and the tricarboxylic acid (TCA) cycle in tolerant cells, the amount of Fru6P in tolerant cells was quantified by high-performance liquid chromatography (HPLC). Upon starvation for 24 h, the amount of Fru6P in the tolerant sub-population tested was 0.02 μ M; however, the level was found to increase \sim 20-fold in the presence of GlcNAc (0.42 μ M) (Figures 5B, 5C, and S5). This sharp increase in the amount of Fru6P in the cytosol of tolerant cells indicated that supplementation of exogenous GlcNAc stimulated its own catabolism in such cells.

To further explore the relationship between GlcNAc catabolization and tolerant cell re-sensitization, we tested the effect of GlcNAc on the $\Delta nagB$ mutant. Unlike the wild-type cell, the survival rate of tolerant cells of the $\Delta nagB$ mutant did not decrease upon treatment with the GlcNAc and ampicillin combination, indicating that cells remained tolerant to ampicillin if GlcNAc catabolism was disturbed (Figure 5A). The GlcNAc re-sensitization effect was also not observable in the stationary phase-induced tolerant cells of the $\Delta nagB$ mutant re-suspended in spent media (Figure S2D). Although GlcNAc catabolism is essential for its ability to re-sensitize cells to ampicillin, the state of glycolysis or TCA cycle upon supplementation of GlcNAc, and whether these processes are involved in the re-sensitization effect of GlcNAc, remain unknown. NADH is the major product of glycolysis and TCA cycle and is a reducing agent directly involved in energy production by donating electrons in the electron transport chain to produce ATP (Saraste, 1999). We then tested the amount of NADH in the tolerant cells in the presence of GlcNAc and found that the level of NADH was about three times higher than that recorded in the absence of GlcNAc (Figure 5D). The increasing amount of NADH in tolerant cells indicated that glycolysis and TCA cycle could be activated by GlcNAc. We then tested whether deletion of either one or both genes that encode NADH dehydrogenase (NADH dehydrogenase I [$\Delta nuoI$], NADH dehydrogenase II [$\Delta ndhI$], and both NADH dehydrogenases [$\Delta ndhnuoI$]), which play an essential role in ATP production, affected the tolerance-suppressing effect of GlcNAc. We found that tolerant cells of the $\Delta ndhnuoI$ mutant could not be eradicated by the GlcNAc and ampicillin combination, as the size of the tolerant population of this strain remained similar to that treated by ampicillin alone ($\sim 10^7$ CFU/mL). However, the re-sensitization effect of GlcNAc could still be observed upon deletion of a single NADH dehydrogenase gene (Figure 5E), suggesting that activation of glycolysis and TCA cycle via enhancement of NADH production and dehydrogenation was an essential step in the GlcNAc re-sensitization process.

ATP is the final product of electron transport chain reaction, and it is also required for peptidoglycan synthesis (Munshi et al., 2013; Saraste, 1999). We hypothesized that the reason why GlcNAc could not

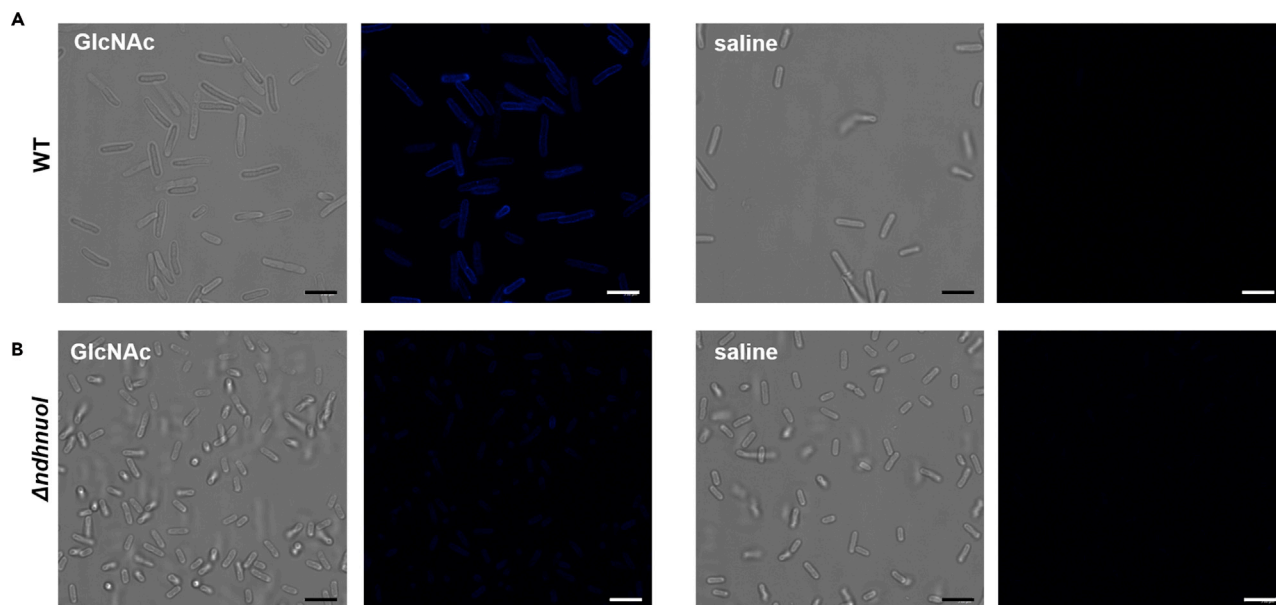


Figure 4. Confocal Microscopic Images of HADA-Labeled Newly Synthesized Peptidoglycan in *E. Coli*

(A and B) Wild-type (WT) *E. coli* (BW25113) (A) and $\Delta ndhnuol$ strain (B) were subjected to 24-h starvation in the presence or absence of GlcNAc. The left and right panels are the bright-field and fluorescence images, respectively (scale bars, 5 μ m).

re-sensitize the tolerant cells of the $\Delta ndhnuol$ mutant was that peptidoglycan synthesis was halted as energy production was limited due to the disruption of NADH dehydrogenation. Therefore, we determined whether peptidoglycan could be synthesized upon supplementation of GlcNAc in $\Delta ndhnuol$. The fluorescence intensity in the $\Delta ndhnuol$ mutant as detected in HADA-labeling experiment was much lower than that of the wild-type in the presence of GlcNAc, indicating that GlcNAc-induced reactivation of peptidoglycan synthesis was blocked upon deletion of the *ndh* and *nuol* genes (Figure 4B). This finding strongly suggests that ATP synthesis is required for activation of peptidoglycan synthesis, which in turn allows tolerant cells to be switched back to the β -lactam-susceptible mode. Apparently GlcNAc can serve as a substrate for glycolysis, as well as peptidoglycan synthesis.

cAMP Regulation and ROS Were Not Involved in GlcNAc Re-sensitization

Previous studies reported that GlcNAc elicited onset of the cAMP signaling pathway, which in turn induced morphological changes, ROS generation, and eventually triggered rapid cell death in *C. albicans* (Castilla et al., 1998; Huang et al., 2010). It was also reported that cAMP played a role in regulating the level of *E. coli* tolerance to β -lactams by mediating oxidative stress response and SOS-dependent DNA repair (Molina-Quiroz et al., 2018). Based on these previous findings, we hypothesized that the cAMP signal-dependent regulation and ROS might also be involved in GlcNAc re-sensitization. To test this hypothesis, we measured the cell survival rate of tolerant cells of the Δcrp , $\Delta cyaA$, and $\Delta cpdA$ gene knockout mutants upon treatment with the GlcNAc and ampicillin combination. The *cyaA* gene encodes the cAMP synthase; the *cpdA* gene product is a cAMP-specific phosphodiesterase that hydrolyzes cAMP; the *crp* gene encodes the cAMP receptor protein (Berkowitz et al., 1980; Imamura et al., 1996). However, our results showed that the synergistic antimicrobial effect of the GlcNAc and ampicillin combination could still be exerted on tolerant cells of the Δcrp , $\Delta cyaA$, and $\Delta cpdA$ mutants, indicating that cAMP-mediated regulations were not involved in the GlcNAc re-sensitization process (Figure 6A). To determine whether ROS was involved in the killing of the tolerant sub-population by ampicillin in the presence of GlcNAc, we used 3'-p-hydroxyphenyl fluorescein (HPF) to measure the level of hydroxyl radicals (\cdot OH) before and after treatment with the GlcNAc and ampicillin combination and found that the amount of hydroxyl radicals remained unchanged upon treatment with GlcNAc and ampicillin (Figure 6B). We also performed 2',7'-dichlorofluorescein diacetate (DCFDA) staining to detect total cellular ROS level and found that GlcNAc-treated cells exhibited the same level of ROS as the untreated tolerant cells (Figure 6C). These findings suggest that GlcNAc does not induce ROS production and that ROS is not involved in the killing of tolerant cells by the GlcNAc and ampicillin combination.

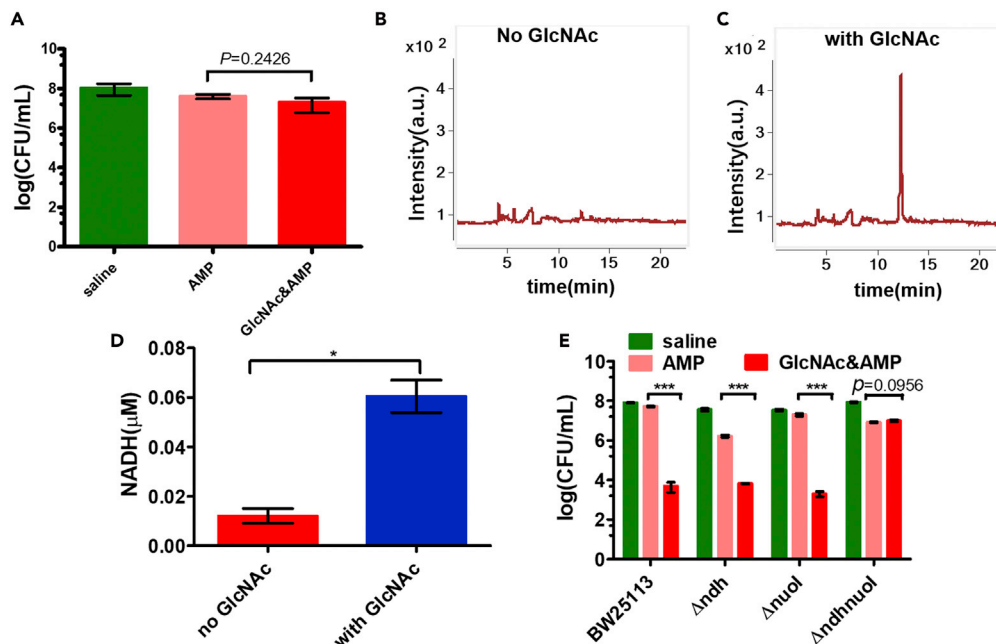


Figure 5. Exogenous GlcNAc Promotes Glycolysis and Significantly Enhances Sensitivity of Tolerant *E. coli* Sub-population to β -Lactam

(A–C) (A) Starved $\Delta nagB$ mutant strains were incubated with ampicillin for 24 h in the presence or absence of GlcNAc (1 mM); cells re-suspended in saline were included as negative control. Intracellular amount of Fru6P in the absence (B) or presence (C) of GlcNAc. Chromatograms of the LC/MS analysis of Fru6P in the samples are presented (m/z of 259 to 79, collision energy 25 V, and retention time of 12.2 min in the HPLC column).

(D) NADH level in the presence or absence of GlcNAc.

(E) Variation in population size of *E. coli* BW25113, $\Delta nuoI$, Δndh , and $\Delta ndhnuoI$. Strains were incubated with ampicillin for 24 h in the presence or absence of GlcNAc; strains treated with saline only were included as negative control. Results are displayed as mean \pm SD. The degree of significance in difference between results of the test samples were determined by Student's t test (ns, no significance; * $p < 0.1$; *** $p < 0.001$).

See also [Figure S5](#)

DISCUSSION

Bacterial antibiotic tolerant sub-populations, the main culprit of chronic and recurrent infections, have been identified in almost every bacterial species. Currently, research reports on tolerant cells mostly focus on identification of genes and metabolic pathways involved in tolerance formation without providing sufficient knowledge that allows design of an effective strategy for eradication of such cells (Harms et al., 2016). In addition, factors that trigger formation of tolerance are not well-defined. Nutrient starvation is one common condition known to strongly elicit onset of stress tolerance in bacteria (Fung et al., 2010). In this work, we aimed to identify non-toxic compounds that can alter the metabolome of physiologically dormant tolerant cells so that they become partially awakened and physiologically reverted back to the antibiotic-susceptible status.

In this study, we first performed high-throughput screening of known nutrients that could suppress phenotypic tolerance and found that GlcNAc and GlcN exhibited the most pronounced effect in converting tolerant cells back to the susceptibility status. Based on results of the gene knockout, HADA labeling and microscopy imaging experiments, we propose a re-sensitization mechanism: uptake of GlcNAc or GlcN by tolerant cells allows them to switch from dormancy to a metabolically active state in which peptidoglycan biosynthesis resumes (Figure 7). Allison et al. previously reported that carbon metabolites, such as glucose, fructose, and mannitol, could significantly enhance the susceptibility of the tolerant sub-population to aminoglycosides via activation of glycolysis, generation of PMF, and subsequently, enhancement of aminoglycoside uptake (Allison et al., 2011). More recently, a phenomenon known as trehalose-catalytic shift in *M. tuberculosis*-tolerant cells was reported; such tolerant cells were found to utilize trehalose as an internal carbon source to synthesize central carbon metabolism intermediates involved

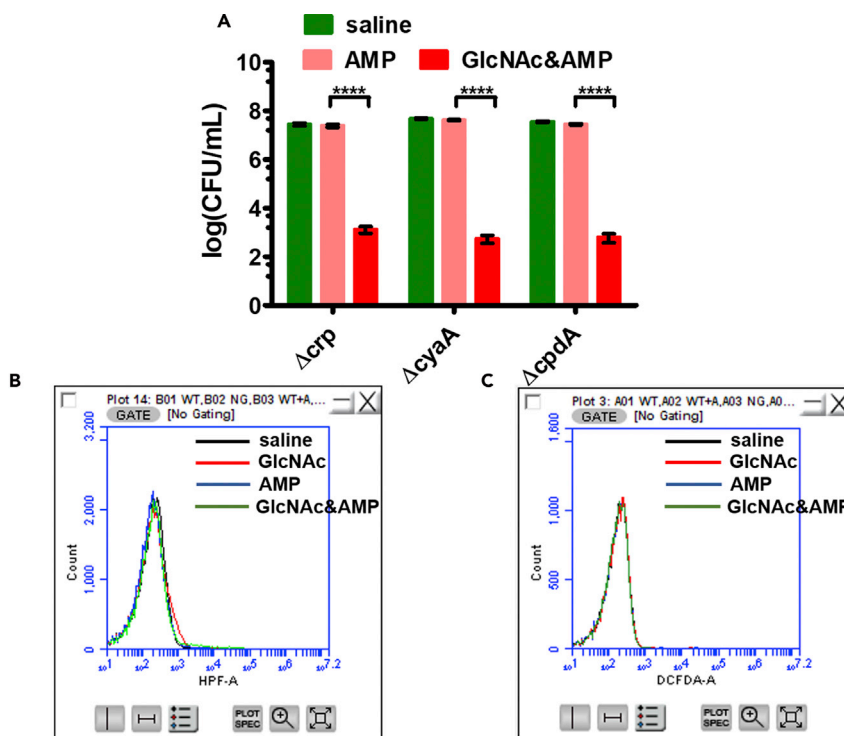


Figure 6. cAMP-Mediated Regulation and ROS Production Were Not Involved in the GlcNAc Re-sensitization Process

(A) Survival rate of three gene knockout strains Δcrp , $\Delta cyaA$, and $\Delta cpdA$ subjected to starvation by washing and re-suspending in saline and then treatment with ampicillin for 24 h in the presence or absence of GlcNAc. Strains treated with saline only were included as negative control.

(B and C) The level of intracellular ROS in the tolerant cells as measured by flow cytometry using two different fluorescence probes of different affinities to free radicals. Starved *E. coli* strain BW25113 was treated with ampicillin, GlcNAc, or a combination of ampicillin and GlcNAc for 24 h before staining with HPF to quantify hydroxyl radical ($\cdot OH$) (B), or with DCFDA to quantify total cellular ROS (C).

in glycolysis and pentose phosphate pathway. Inhibitor of trehalose-catalytic shift process could be used as an adjuvant to potentiate antibiotic efficacy by interfering with these adaptive strategies (Lee et al., 2019). Apparently, shutdown of carbon metabolism and biosynthetic activities is a key step in tolerance formation and is also essential for maintenance of the tolerance phenotype. Hence interference by exogenous carbon sources can drastically alter the level of tolerance of bacteria to β -lactam drugs. As a carbon metabolite, GlcNAc can activate glycolysis and kick start the TCA cycle in tolerant cells as the levels of both Fru6P and NADH were found to have increased upon supplementation of GlcNAc. The finding that the GlcNAc re-sensitization effect was abolished if the NADH dehydrogenase genes were deleted indicates that glycolysis and assimilation of NADH by the electron transport chain are essential steps in the GlcNAc re-sensitization process or peptidoglycan synthesis (Figure 7). Our data therefore show that the underlying mechanism by which GlcNAc can re-sensitize bacterial tolerant cells to ampicillin involves eliciting glycolysis and allowing peptidoglycan biosynthesis to resume.

GlcNAc is known to induce cell death in *C. albicans* by inducing alteration in mitochondrial metabolism and activating the cAMP-regulated pathways, which in turn leads to accumulation of ROS and cell death (Du et al., 2015). Our data showed that the underlying mechanism of GlcNAc-mediated tolerant cell killing in *E. coli* differs from that in *C. albicans*, as GlcNAc alone can induce cell death in *C. albicans* due to its ability to cause ROS accumulation, whereas it cannot do so in *E. coli*. GlcNAc can also trigger an increase in cAMP level in *C. albicans* (Castilla et al., 1998). The signaling molecule cAMP plays an important role in regulation of the *nag* regulon, which contains genes whose products play a role in GlcNAc and GlcN metabolism in *E. coli*, and was reported to be involved in tolerance formation (Castilla et al., 1998; Molina-Quiroz et al., 2018; Plumbridge, 1990). However, we found that the GlcNAc re-sensitization effect was not affected by

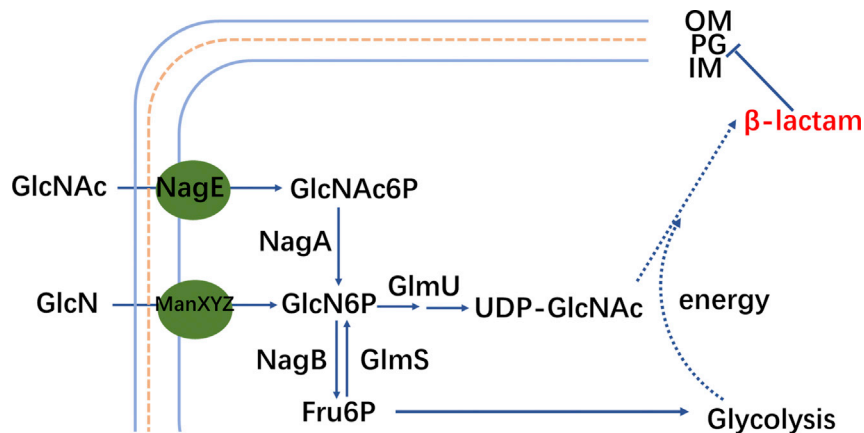


Figure 7. Proposed Mechanism of GlcNAc-Mediated Sensitization of Starvation-Inducible Bacterial Tolerant Sub-population to β -Lactams

GlcNAc/GlcN is transported into the cell membrane and becomes phosphorylated to generate GlcNAc6P, which was subsequently converted to UDP-GlcNAc and Fru6P, the precursor molecules for peptidoglycan synthesis and initiation of glycolysis, respectively. ATP produced by glycolysis is required for peptidoglycan synthesis. Initiation of peptidoglycan synthesis in tolerant cells under starvation stress renders them susceptible to β -lactam. OM, outer membrane; PG, peptidoglycan; IM, inner membrane.

deletion of the *crp*, *cyaA*, and *cpdA* genes, suggesting that cAMP signaling is not involved in GlcNAc-re-sensitization of the tolerant sub-population to ampicillin, and that the bactericidal/re-sensitization effects of GlcNAc on bacteria and fungus involve activation of different cellular mechanisms.

GlcNAc and GlcN are known to exhibit beneficial pharmacological effects in treatment of osteoarthritis (Kubomura et al., 2017). The therapeutic potential of GlcNAc is mainly attributed to its anti-inflammatory and chondroprotective effects (Dalirfardouei et al., 2016). GlcNAc also shows promising therapeutic efficacy in treatment of chronic inflammatory bowel disease by restoring the intestinal matrix and improving the epithelial morphology (Salvatore et al., 2000). As GlcNAc is non-toxic, this compound should be further explored to determine its potential of being used as an antibiotic adjuvant for treatment of chronic and recurrent infections in immunocompromised patients caused by members of Enterobacteriaceae.

The experimental system reported here demonstrates a novel approach to restore the antibiotic susceptibility phenotype of bacterial tolerant cells by using GlcNAc as a β -lactam adjuvant. Findings in this work broaden the current understanding of tolerance physiology by demonstrating that activating specific metabolic pathways of tolerant cells can significantly enhance the susceptibility of tolerant cells to specific antibiotic.

Limitations of the Study

Our study demonstrated that supplementation of exogenous GlcNAc could effectively re-sensitize starvation-induced tolerant bacterial sub-population to β -lactams but not aminoglycosides and fluoroquinolones, indicating that the re-sensitization effect of GlcNAc is confined to activation of peptidoglycan synthesis. The cellular basis of this phenomenon needs to be investigated. At this stage we have only screened carbon-based nutrients. It remains to be seen whether supplementation of other types of nutrients, such as those of nitrogen and phosphorus sources, may also affect bacterial tolerance, and to what extent. On the other hand, the re-sensitization effect of GlcNAc has to be further tested in animal models as there may be factors impeding penetration of the compound into tolerant bacterial cells, especially those residing in phagocytes and those under conditions such as hypoxic stress, which may affect peptidoglycan synthesis and glycolysis. In other words, the clinical application potential of GlcNAc has to be evaluated.

Resource Availability

Lead Contact

Further information and requests for resources should be directed to and will be fulfilled by the Lead Contact, Sheng Chen (shechen@cityu.edu.hk).

Materials Availability

This study did not generate new unique reagents.

Data and Code Availability

All the data are available within the article.

METHODS

All methods can be found in the accompanying [Transparent Methods supplemental file](#).

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.isci.2020.101740>.

ACKNOWLEDGMENTS

This study was funded by the Collaborative Research Fund from the Research Grant Council of the Government of Hong Kong SAR (C5026-16G) and the Research Impact Fund (R5011-18F).

AUTHOR CONTRIBUTIONS

M.M.W. designed and performed the study; E.W.C.C. designed the study and edited the manuscript; Y.C. and K.C.C. helped with animal experiments; P.-k.S. helped with mass spectrum analysis; S.C. designed the study, supervised the whole project, and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: June 2, 2020

Revised: September 12, 2020

Accepted: October 23, 2020

Published: November 20, 2020

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iScience, Volume 23

Supplemental Information

***N*-Acetyl-D-Glucosamine Acts as Adjuvant that Re-Sensitizes Starvation-Induced Antibiotic-Tolerant Population of *E. Coli* to β -Lactam**

Miaomiao Wang, Edward Wai Chi Chan, Chen Yang, Kaichao Chen, Pui-kin So, and Sheng Chen

Negative control 0.376±0.023	L-Arabinose 0.405±0.018	N-Acetyl-D-Glucosamine 0.055±0.009	D-Saccharic Acid 0.159±0.015	Succinic Acid 0.096±0.009	D-Galactose 0.104±0.012	L-Aspartic Acid 0.104±0.013	L-Proline 0.085±0.013	D-Alanine 0.071±0.014	D-Trehalose 0.075±0.016	D-Mannose 0.161±0.014	Dulcitol 0.174±0.015
D-Serine 0.072±0.013	D-Sorbitol 0.068±0.011	Glycerol 0.072±0.005	L-Fucose 0.088±0.014	D-Gluconic Acid 0.077±0.013	D-Gluconic Acid 0.069±0.009	D,L-α-Glycerol Phosphate 0.182±0.015	D-Xylose 0.448±0.009	L-Lactic Acid 0.095±0.008	Formic Acid 0.242±0.013	D-Mannitol 0.073±0.011	L-Glutamic Acid 0.252±0.007
D-Glucose-6-Phosphate 0.071±0.014	D-Galactonic Acid-γ-Lactone 0.121±0.010	D,L-Malic Acid 0.117±0.016	D-Ribose 0.39±0.015	Tween 20 0.073±0.008	L-Rhamnose 0.14±0.015	D-Fructose 0.059±0.01	Acetic Acid 0.391±0.016	α-D-Glucose 0.077±0.011	Maltose 0.071±0.016	D-Melibiose 0.165±0.015	Thymidine 0.134±0.019
L-Asparagine 0.104±0.014	D-Aspartic Acid 0.23±0.015	D-Glucosaminic Acid 0.183±0.009	1,2-Propanediol 0.137±0.016	Tween 40 0.108±0.018	α-Keto-Glutaric Acid 0.116±0.013	α-Keto-Butyric Acid 0.119±0.009	α-Methyl-D-Galactoside 0.12±0.015	α-D-Lactose 0.123±0.014	Lactulose 0.161±0.017	Sucrose 0.126±0.012	Uridine 0.106±0.008
L-Glutamine 0.122±0.01	m-Tartaric Acid 0.214±0.009	D-Glucose-1-Phosphate 0.068±0.013	D-Fructose-6-Phosphate 0.065±0.012	Tween 80 0.118±0.011	α-Hydroxy Glutaric Acid-γ-Lactone 0.224±0.016	α-Hydroxy Butyric Acid 0.07±0.014	β-Methyl-D-Galactoside 0.127±0.018	Adonitol 0.143±0.016	Maltotriose 0.071±0.008	2-Deoxy Adenosine 0.066±0.01	Adenosine 0.137±0.015
Glycyl-L-Aspartic Acid 0.105±0.016	Citric Acid 0.101±0.013	m-Inositol 0.144±0.011	D-Threonine 0.079±0.014	Fumaric Acid 0.105±0.016	Bromo Succinic Acid 0.084±0.017	Propionic Acid 0.099±0.009	Mucic Acid 0.099±0.01	Glycolic Acid 0.24±0.018	Glyoxylic Acid 0.237±0.020	D-Cellobiose 0.113±0.014	Inosine 0.074±0.010
Glycyl-L-Glutamic Acid 0.103±0.016	Tricarballic Acid 0.191±0.016	L-Serine 0.074±0.010	L-Threonine 0.102±0.014	L-Alanine 0.081±0.018	L-Alanyl-Glycine 0.081±0.016	Acetoacetic Acid 0.508±0.011	N-Acetyl-β-D-Mannosamine 0.082±0.013	Mono Methyl Succinate 0.114±0.014	Methyl Pyruvate 0.096±0.015	D-Malic Acid 0.202±0.020	L-Malic Acid 0.098±0.017
Glycyl-L-Proline 0.116±0.011	p-Hydroxy Phenyl Acetic Acid 0.173±0.019	m-Hydroxy Phenyl Acetic Acid 0.167±0.015	Tyramine 0.251±0.019	D-Psicose 0.15±0.012	L-Lyxose 0.553±0.011	Glucuronamide 0.109±0.009	Pyruvic Acid 0.114±0.008	L-Galactonic Acid-γ-Lactone 0.095±0.014	D-Galacturonic Acid 0.152±0.016	Phenylethylamine 0.21±0.014	2-Aminoethanol 0.217±0.016

Figure S1. Optical density (OD₆₀₀) of culture re-grown from the tolerant cell fraction in the PM plate that survived ampicillin treatment, Related to Figure 1. Bacteria subjected to complete nutrient starvation by washing and incubating in saline (0.85% NaCl) were added to each well of the indicated PM plate, followed by treatment with ampicillin for 4hr. The cell suspension containing the tolerant sub-population that survived ampicillin treatment was then diluted into LB broth and incubated at 37°C for 5 hrs. The cell density of the culture derived from this re-grown tolerant sub-population was expected to be proportional to the size of the sub-population that survived ampicillin treatment in the presence of various test compounds. A lower cell density infers a higher strength of the compound that re-sensitize the tolerant sub-population to ampicillin. Black, OD₆₀₀ of regrowth cells < 0.06; dark grey, OD₆₀₀ of regrowth cells < 0.07; light grey, OD₆₀₀ of regrowth cells < 0.08. Error bars indicate the standard errors of the means.

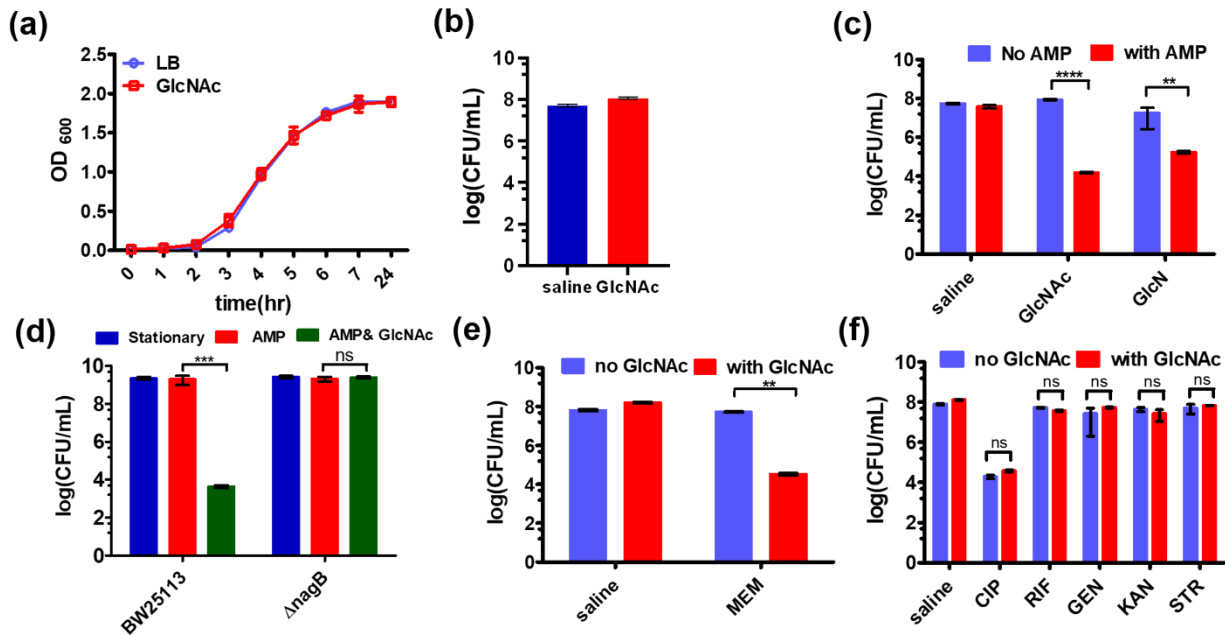


Figure S2. Effect of GlcNAc on sensitivity of *E. coli* tolerant cells to different antibiotics, Related to Figure 1. (a) Comparison of growth rate of *E. coli* strain BW25113 with or without GlcNAc (1mM) in LB broth shows that GlcNAc is non-toxic and does not inhibit cell growth. (b) Comparison of population size of *E. coli* strain BW25113 re-suspended in saline and incubated for 24 hrs with or without supplementation of GlcNAc (1mM). (c) Treatment of starved *E. coli* strain BW25113 with ampicillin for 24hrs in the presence or absence of GlcNAc (1mM) or GlcN(1mM). (d) Treatment of stationary phase *E. coli* strain BW25113 and Δ nagB with ampicillin for 4hrs in the presence or absence of GlcNAc (1mM) in the spent media (e) Treatment of starved clinical *E. coli* strain with meropenem (40 μ g/mL) for 24hrs in the presence or absence of GlcNAc (1mM). (f) Starved *E. coli* were incubated with different antibiotics for 24hrs in the presence or absence of GlcNAc (1mM). CIP, ciprofloxacin (2 μ g/mL); RIF, rifampin (25 μ g/mL); GEN, gentamycin (10 μ g/mL); KAN, kanamycin (30 μ g/mL); STR, streptomycin (50 μ g/mL). Results are displayed as mean \pm SD; the degree of significance in the differences between the control and test samples, as determined by Student's test, is indicated (ns, no significance; * P <0.1; ** P <0.01; *** P <0.001; **** P <0.0001). Three biological repeats were carried out.

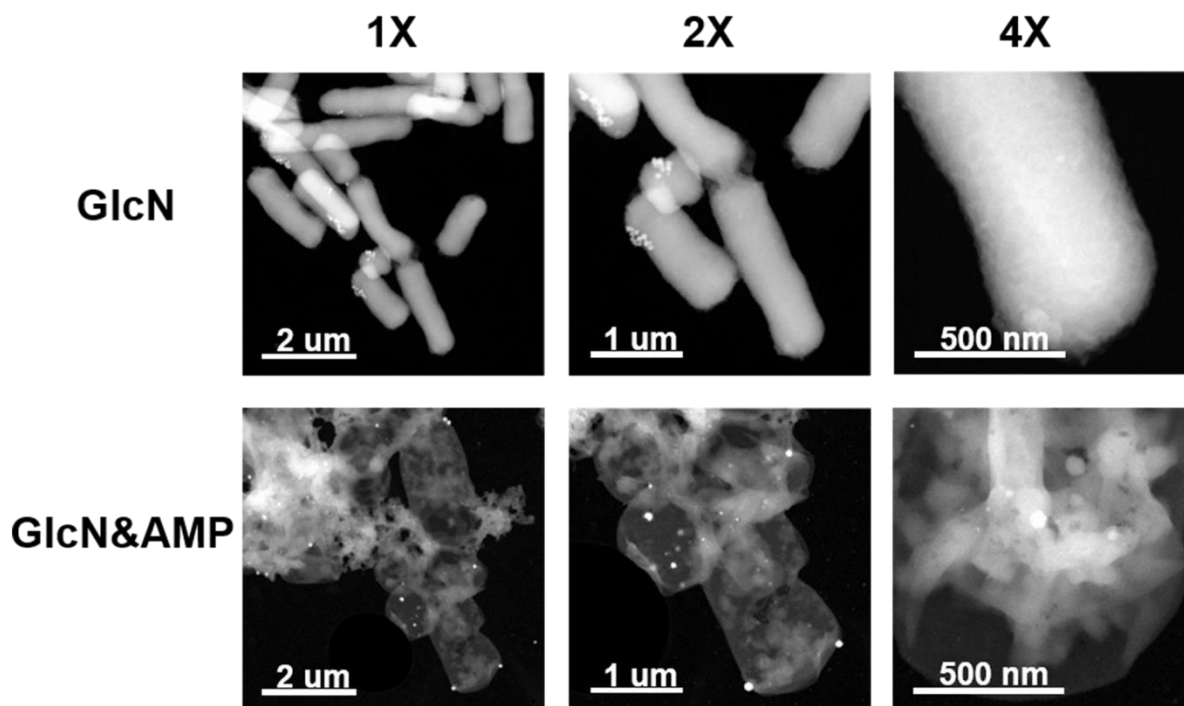


Figure S3. Starved cells were incubated with GlcN(1mM) or GlcN plus ampicillin for 24hrs before taking SEM image, Related to Figure 2.

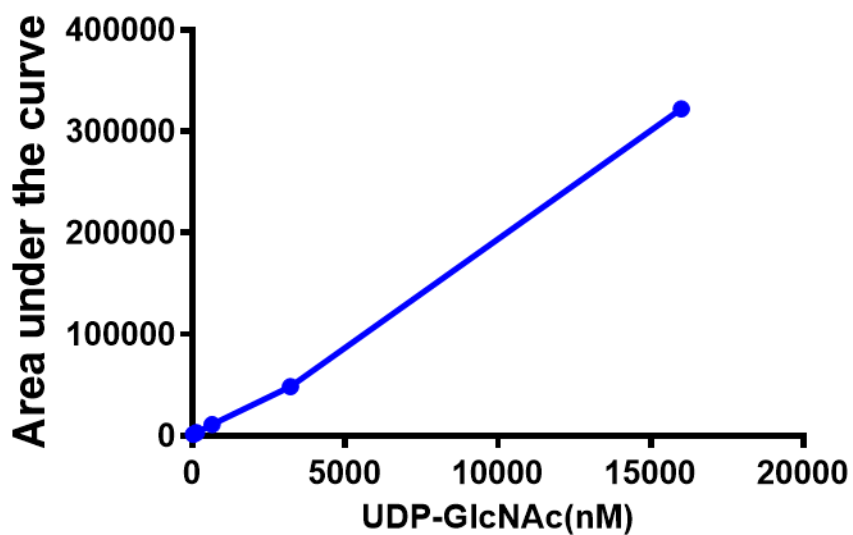


Figure S4. Standard curve of UDP-GlcNAc, Related to Figure 3. 2μL of gradient-diluted standard with concentrations ranging from 25.6nM to 16μM were analyzed by HPLC-MS operated in negative ion mode. The area under the curve of each sample was determined by GraphPad Prism.

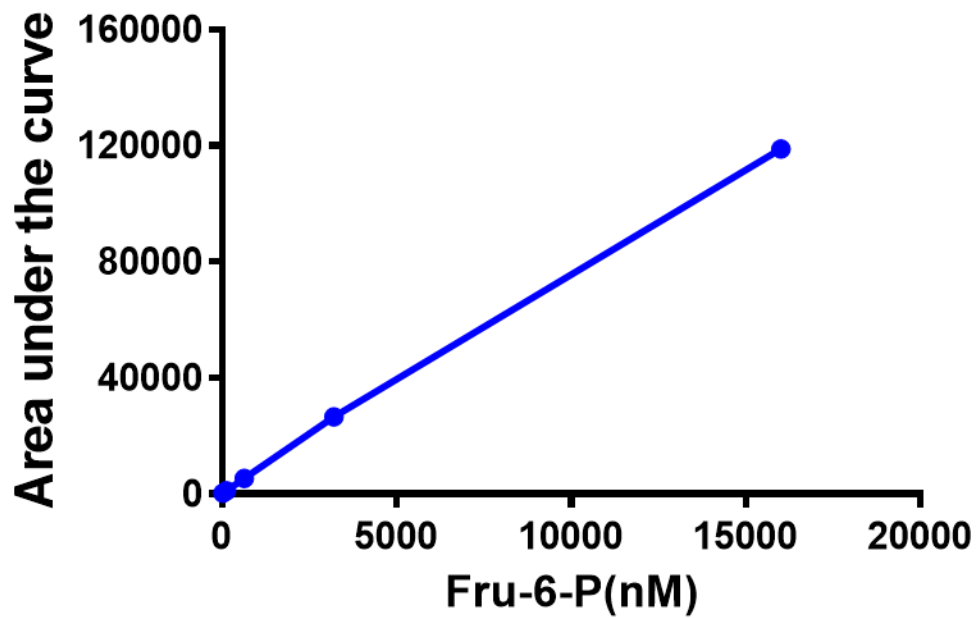


Figure S5. Standard curve of fructose-6-P, Related to Figure 5. 2 μ L of gradient-diluted standard with concentrations ranging from 5.12nM to 16 μ M were analyzed by HPLC-MS operated in negative ion mode. The area under the curve of each sample was determined by GraphPad Prism.

TRANSPARENT METHODS

Strains, culture conditions and chemicals

Escherichia coli strain BW25113 and clinically isolated *E. coli* strain (strain 2) were used in this work, Luria-Bertani (LB) broth was used for all cultures. All the single knockout mutants ($\Delta nagA$, $\Delta nagB$, Δndh , $\Delta nuol$, Δcrp , $\Delta cyaA$, $\Delta cpdA$) were obtained from the Keio collection of *E. coli* BW25113 (Baba et al., 2006). $\Delta ndhnuol$ was constructed by lambda red recombination, using plasmid PKD46 to replace the *nuol* gene with the chloramphenicol resistance gene in the parent strain of Δndh . Standard LB agar (Difco, Leeuwarden, The Netherlands) was used in antibiotic assays. All the antibiotics used in this work, as well as GlcNAc, GlcN, Fru6P and UDP- GlcNAc were purchased from Sigma-Aldrich. HADA was synthesized following the procedure described by Kuru et al. (Kuru et al., 2015). PM plates were purchased from Biolog.

Tolerance assays

Upon reaching the exponential phase ($OD_{600}=0.2$), bacteria were deprived of all essential nutrients by washing and re-suspending in saline (0.85% NaCl). The test population was then treated with GlcNAc (1mM) / GlcN (1mM) with or without ampicillin (100 μ g/ml) for 24 hrs at 37 °C under constant shaking (250rpm/min). Standard serial dilution and plating on LB agar was performed to determine the fraction of the test population that survived the treatment (Fung et al., 2010).

Mouse deep-seeded thigh infection model

For all animal experiments, six-weeks-old NIH male mice (body weight, ~20g) were purchased from the Guangdong Center for experimental animals and allowed for food and water throughout the study. The NIH mice were made neutropenic by administering 150mg/kg cyclophosphamide at 3 days and 1 day before infection, respectively. An inoculum of 1×10^7 *E. coli* BW25113 were injected into the two thighs of mouse. At 24 hrs post-infection, the mice received antibacterial treatment (i.p.) every 12 hrs for 72hrs, with a dosage of 20mg cefotaxime/kg and 1mmol GlcNAc /kg. The mice were euthanized and the infected thighs were aseptically excised, homogenized in saline and the number of *E. coli* was enumerated by

serial dilution and plating on LB plate. Comparison between different treatment groups were analyzed using one-way ANOVA and post hoc Tukey test and presented by Graph Pad Prism. All experimental protocols followed the standard operating procedures of the approved biosafety level 2 animal facilities and were approved by the Animal Ethics Committee of The Hong Kong Polytechnic University.

Electron microscopy

Cells treated with GlcNAc, GlcN, ampicillin or saline (negative control) for 24hrs were visualized by scanning electron microscopy (SEM). Cells were fixed in 0.4% polyoxymethylene overnight and O_3 for 2hrs, then washed 3 times with PBS. The cells were dehydrated using pure ethanol, and then infiltrated and embedded in Spurr resin.

Analysis of accumulation products by LC/MS

The bacterial cytosolic sample was analyzed by an electrospray ionization triple quadrupole mass spectrometer (Agilent), with a 6460 liquid chromatography (Agilent). Upon treatment with GlcNAc alone for 24hrs, bacteria were centrifuged at 6500xg for 10mins. The cells were then re-suspended with 1mL B-PER and vortexed for 15mins to achieve cell lysis; cell debris was removed and 5 volumes of acetone were added to cause precipitation of macromolecules. The insoluble part was separated by centrifugation for 10 mins at 16000xg. The supernatant was transferred to a microtube and dried at 56 °C in a vacuum concentrator for 2hrs. The pellet was dissolved in 50µL of water before LC/MS analysis and 2µL were injected into an Acquity UPLC BEH HILIC column (150 by 4.6 mm; Waters) for measurement of the amount of UDP- GlcNAc, and a NH₂ column (150 by 2mm; Phenomenex) for Fru6P, using a 20-min-gradient program for UDP- GlcNAc and 22-min-gradient program for Fru6P, setting a flow rate of 0.2ml/min. The column was subjected to 5 minutes of washing with 100% buffer A (5mM ammonium formate with 5% ammonium hydroxide for UDP- GlcNAc; 20mM ammonium acetate with 5% ammonium hydroxide for Fru6P), followed by a linear gradient with 40% buffer B (5mM ammonium formate with 95% acetonitrile for UDP- GlcNAc; 100% acetonitrile for Fru6P) . The electrospray ionization triple quadrupole mass spectrometer was operated in negative ESI MRM mode. The following MRM transitions were used for UDP- GlcNAc: m/z 606.2 to 385 (quantifier), collision energy 25V, for Fru6P: m/z 259 to 79 (quantifier)

collision energy 25V. A series of dilutions of standard Fru6P and UDP- GlcNAc were also analyzed by LC-MS to create a standard curve for determining the intracellular concentration of Fru6P and UDP- GlcNAc.

NADH measurement

E. coli was washed and re-suspended in saline until a concentration of OD₆₀₀ of 0.2 was reached, followed by incubation with GlcNAc for 24hrs. Cell pellets were washed and re-suspended with NADH extraction buffer, followed by determination of the concentration of NADH by using the the EnzyChrom NAD/NADH Assay Kit (BioAssay Systems).

Fluorescence-labelling of peptidoglycan

Log-phase culture of the test *E. coli* strain was washed and re-suspended in saline to produce a cell suspension, followed by incubation with GlcNAc for 24hrs. The cell suspension was then supplemented with 500µM HADA (Ex 405nm/Em 460nm) and incubated for 30mins. The cells were then fixed in 0.4% polyoxymethylene for 2hrs, washed 3 times with PBS, and then visualized by a Leica TCS SP8 MP Multiphoton Microscope; the microscopy images were analyzed by the LAS X software.

ROS measurement

Log-phase culture of the test *E. coli* strain was washed and re-suspended in saline, followed by incubation with GlcNAc for 24hrs. The cells were then incubated with 5mM HPF (Molecular Probes) or DCFDA(Invitrogen) for 30mins, and then washed twice in PBS to remove the excess dye. HPF is a probe highly specific for ·OH, whereas DCFDA was used to measure the total ROS level. Fluorescence was measured at an excitation wavelength of 485nm and an emission wavelength of 535nm by flow cytometry (BD Accuri C6), the cells were identified according to the FSC (forward scatter) and SSC (side scatter) parameters.

Screening of compounds that suppress starvation-induced antibiotic tolerance

Upon reaching the exponential phase ($OD_{600}=0.2$), bacteria were washed and deprived of all essential nutrients by re-suspending in saline. 180 μ L of this cell suspension was added to each well of the PM plates (Biolog), followed by addition of ampicillin to produce a final concentration of 100 μ g/ml. The plate was incubated at 37 $^{\circ}$ C for 24 hours under constant shaking (250rpm/min). 1 μ L of the cell suspension was taken from each well of the PM plates and then mixed with 99 μ L LB broth in the corresponding well in a reporter plate and incubated at 37 $^{\circ}$ C for 5hrs. The cell density, which was proportional to the number of viable cells and hence the tolerance level of cells in the 1 μ L inoculum, was determined by measuring the OD_{600} .

SUPPLEMENTAL REFERENCES

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