Current Microbiology (2025) 82:278
https://doi.org/10.1007/s00284-025-04261-7

Coordinated Transcriptional Increases in Cell Wall Synthesis Genes in *Neisseria gonorrhoeae* Lacking the Lytic Transglycosylase, *ltgA*

Candra O. Broadie[1] · Alaa I. Telchy[1] · Antonio T. Baines[1] · Joseph P. Dillard[2] · Robert A. Nicholas[3] · Daniel Williams[4]

Received: 21 November 2024 / Accepted: 25 April 2025

Abstract

Lytic transglycosylase A in *Neisseria gonorrhoeae* cleaves the *β*-1,4-glycosidic bond between peptidoglycan (PG) monomers to liberate 1,6-anhydro-PG fragments that are either recycled or released as cytotoxic fragments. To gain further insight into the effect of LtgA on cellular processes in *Neisseria gonorrhoeae*, we performed a proteomic analysis comparing wild-type and an isogenic *ltgA* null mutant strain. Proteins were separated by two-dimensional gel electrophoresis and identified by MALDI-TOF mass spectrometry, which revealed several proteins that were increased in their level of expression upon loss of LtgA. The most notable changes corresponded to enzymes related to aminosugar and pyrimidine metabolism. Quantitative real-time RT-PCR of mRNA from a *ltgA* null strain confirmed increased transcription of genes encoding enzymes involved in UDP-N-acetylglucosamine (UDP-GlcNAc) synthesis, a major precursor in PG and lipooligosaccharide (LOS) synthesis, during normal growth conditions and following exposure to penicillin. We also found that the *ltgA* mutant strains were more susceptible to β-lactam antibiotics, vancomycin, and the human-cathelicidin antibacterial peptide, LL-37, than their corresponding wild-type parental strains. Our results suggest that increased expression of enzymes responsible for production UDP-GlcNAc is an adaptive response due to inactivation of *ltgA* and/or exposure to penicillin.

✉ Daniel Williams
dawilliams@alasu.edu

Candra O. Broadie
cbroadie0157@yahoo.com

Alaa I. Telchy
atelchy@gmail.com

Antonio T. Baines
abaines@nccu.edu

Joseph P. Dillard
jpdillard@wisc.edu

Robert A. Nicholas
robert_nicholas@med.unc.edu

[1] Department of Biological and Biomedical Sciences, North Carolina Central University, Durham, NC 27707, USA

[2] Department of Medical Microbiology and Immunology, University of Wisconsin-Madison, Madison, WI 53706-1521, USA

[3] Departments of Pharmacology and Microbiology and Immunology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7365, USA

[4] Department of Biological Sciences, Alabama State University, 915 S. Jackson Street, Montgomery, AL 36104, USA

Abbreviations

PG	Peptidoglycan
Pen	Penicillin
MurNAc	N-acetylmuramic acid
GlcNAc	N-acetylglucosamine
GlcN-6-P	Glucosamine-6-phosphate
PGCT	Peptidoglycan-derived cytotoxin fragments

Neisseria gonorrhoeae is a Gram-negative, strict human pathogen that causes the sexually transmitted infection gonorrhea. Several determinants, such as demographics [1], social behavior [2], and biological factors [3, 4], contribute to the prevalence of gonorrhea. Individuals infected with *N. gonorrhoeae* have an increased likelihood of both transmitting and acquiring HIV [5]. With over 106 million infections worldwide per year [6], the treatment and prevention of gonorrhea is an enormous economic burden of global concern, especially with the emergence of antibiotic-resistant strains. Many inexpensive antibiotics traditionally used to treat gonorrhea, e.g. penicillin, tetracycline, fluoroquinolones, and macrolides, are no longer effective because of resistance mechanisms [7]. While most gonococcal infections can be resolved by the current antibiotic regimen (dual therapy with

Published online: 06 May 2025

ceftriaxone and azithromycin or doxycycline in the U.S.) [8], these antibiotics are expensive, not always available in poorer countries, and their minimum inhibitory concentrations have increased markedly in the past decade, especially in the Western Pacific Region countries [9]. If gonococcal infections are left untreated, more invasive forms of the disease can develop, resulting in pelvic inflammatory disease, ectopic pregnancy, infertility, or disseminated gonococcal infections.

Peptidoglycan (PG), a major component of the cell wall and the target of several antibiotics, is an important biological factor that has been implicated in gonococcal pathogenesis [10, 11]. PG is composed of glycan strands containing alternating aminosugars, N-acetylmuramic acid (MurNAc) and N-acetylglucosamine (GlcNAc), connected by a β-1,4-glycosidic bond, with tetrapeptides or pentapeptides linked to the carboxylate moiety of each MurNAc residue. In mature PG, the peptides from two adjacent glycan strands are cross-linked to one another, conferring rigidity, maintenance of cell shape, and resistance to osmotic pressure. The PG layer is continuously remodeled during growth, causing PG fragments to be either recycled or released [12]. A multi-enzyme complex composed of lytic transglycosylases (LTs), murein hydrolases, and synthases has been proposed for PG synthesis and remodeling during bacterial growth [13, 14]. It is possible that the regulation of these enzymatic activities is under strict control to avoid lysis while allowing cellular growth, suggesting that a delicate balance must be maintained between PG turnover and cell wall synthesis [15, 16]. PG remodeling is accomplished in part by the activity of lytic transglycosylases, which are a subclass of murein lyases that cleave the β-1,4-glycosidic bonds between MurNAc and GlcNc, with the concomitant formation of 1,6-anhydromuramic acid residues at the terminal position [17].

The gonococcus has several well-characterized lytic transglycosylases that are involved in PG release and recycling [18, 19], cell separation [20], and chromosomal DNA secretion via a type IV secretion system [21, 22]. Cloud-Hansen et al. [19] determined that the lytic transglycosylases LtgA and LtgD are responsible for the liberation of PG fragments for both recycling and release, with each enzyme producing about half of the released PG fragments. Interestingly, LtgC, which is involved in cell separation, was the only other lytic transglycosylase in *N. gonorrhoeae* shown to produce a slight decrease in PG monomer release compared to wild-type [20]. 1,6-anhydro-PG monomers released by *N. gonorrhoeae* are identical to peptidoglycan-derived cytotoxins (PGCTs) in other Gram-negative bacteria that cause cellular damage [23] and induce inflammatory cytokines [24, 25]. Lytic transglycosylases have been implicated in virulence [10, 26] and in modulating expression of certain cellular processes [15, 27, 28]. Several studies have reported that Gram-negative bacteria with mutations in murein hydrolases have reduced cytoplasmic PG monomer pools owing to changes in PG recycling [19, 29, 30], and specific cytoplasmic proteins have been identified that respond to fluctuations in the level of cytoplasmic UDP-MurNAc-pentapeptide and anhMurNAc-tripeptide in some species [15, 27, 28, 31, 32]. However, it is unclear what changes occur in protein and gene expression in response to changes in PG recycling and release in *N. gonorrhoeae*.

Since LtgA is responsible for the release of approximately 40–50% of PG in *N. gonorrhoeae* [19], we compared the protein expression profiles following normal growth of wild-type and an isogenic *ltgA* mutant to identify those proteins whose expression was altered. We identified 7 proteins whose expression increased upon loss of *ltgA*; four of these are involved in maintaining UDP-GlcNAc levels important for PG and LOS synthesis. Our data further demonstrated that gene transcription of enzymes involved in aminosugar, and pyrimidine metabolism increase markedly in an *ltgA* mutant strain during stationary phase under normal growth conditions and in both wild-type and mutant *ltgA* strains following exposure to a sub-lethal concentration of penicillin. We also determined that MS11 lacking *ltgA* was more sensitive than the parental strain to certain β-lactam antibiotics, to vancomycin, and to a human antibacterial peptide, LL-37, that *N. gonorrhoeae* encounters during infection.

Materials and Methods

Bacterial Strains and Growth Conditions

All experiments were carried out with non-piliated gonococcal variants except when used for transformation experiments, as piliation is required for competence. *N. gonorrhoeae* strains FA19 and MS11, and their respective isogenic *ltgA* deletion strains (Table 1), were grown in 30 ml of GC base liquid (GCBL) medium (1.5% proteose peptone no. 3, 0.4% K_2HPO_4, 0.1% KH_2PO_4, 0.1% NaCl, 0.1% starch; pH 7.2) containing Kellogg's supplements [33] and 0.042% $NaHCO_3$ with aeration or on GC agar plates (Difco) containing supplements in 5% (v/v) CO_2 at 37 °C. MS11 and its isogenic strains, KC100 (MS11 Δ*ltgA*) and KH599 (KC100 complemented with wild-type *ltgA* at a second site in the chromosome), were kindly provided by Dr. Joe Dillard (University of Wisconsin).

Construction of FA19 ΔltgA

Gene-specific primers for *ltgA* (Table S1) were designed based on the genome sequence of FA1090 (GenBank accession no AE004969) and used to amplify *ltgA* from FA19 genomic DNA. The 1848 bp amplified fragment was cloned into the vector pBAD-TOPO (Invitrogen, Carlsbad, CA).

Table 1 Strains and plasmids used in the present study

Strains or plasmid	Relevant characteristics	References
Plasmids		
pBAD-TOPO-T/A	pUC-derived expression vector, Ap^r	Invitrogen
DW2135	*ltgA* disruption plasmid (Kan^r/Ap^r)	This study
FA19	*N. gonorrhoeae* wild-type strain	[36]
LW9279	FA19 transformed with DW2135	This study
MS11	*N. gonorrhoeae* wild-type strain	[18]
KC100	MS11 *ltgA* deletion mutant	[18]
KH599	KC100 (complemented with *ltgA* wt	Dillard, J. P. (unpublished)
KH560	MS11 *ltgA* and *ltgD* deletion mutant	[19]

The resulting plasmid was digested with ClaI, the overhangs filled-in with T4 DNA Polymerase, and the SmaI-digested *aphA*-3 kanamycin-resistance (*kanA*) cassette from plasmid pUC18K was ligated into the digested vector. This construct (designated DW2135; Table 1) was transformed into gonococcal strain FA19 to create mutant LW9279 ($ltgA::Km^R$). LW9279 was confirmed by PCR (data not shown) using *ltgA*- and *kanA*-specific primers (Table S1) and verified by sequence analysis (data not shown).

Two-Dimensional Gel Electrophoresis (2DGE)

Gonococcal strains FA19 and LW9279 were grown in GCBL to mid-log or stationary phase and harvested by centrifugation at 4000 × *g*. A modified 2DGE procedure was performed on proteins isolated from strains. Briefly, cells at the indicated growth phase were resuspended in lysis buffer (10 mM Tris, pH 7.4, 0.3% SDS). An equal volume of urea sample buffer (9.5 M urea, 2% Nonidet P-40, 5% beta-mercaptoethanol, and 2% ampholines consisting of 1.6% pH 5–7 and 0.4% pH 3.5–10) was added to samples prior to 2DGE analysis (Kendrick Laboratories, Inc; Madison, WI). The difference between two corresponding spots was calculated by dividing spot density by the total density of all measured spots. Five spots that were most increased in expression in LW9279 relative to FA19 were excised from the silver-stained gel and identified by mass spectrometry (Protein Chemistry Core Facility, Columbia University).

Antibiotic Susceptibility Assays

Epsilometer (E-test; Biomérieux, Durham, NC) strips were used to evaluate the susceptibility of gonococcal FA19 and MS11 wild-type and isogenic mutant strains to antibiotics. Strains were grown on GC agar plates containing Kellogg supplements at 37 °C, 5% (v/v) CO_2 overnight and then inoculated into GCBL-medium. The cells were harvested between an optical density 550 nm (OD_{550}) of 0.3–0.5, spread onto GC agar plates, and incubated for 30 min. KH599 was spread onto GC agar plates containing 1 mM Isopropyl β-d-1-thiogalactopyranoside (IPTG) to induce expression of the wild-type copy of *ltgA* located at a second site within the chromosome. E-test strips were applied to the surface of the plated cells and incubated for 24 and 48 h. Plates containing *E*-test strips were read under a dissecting microscope by two independent viewers. All assays were carried out in triplicate and the averages of several assays were calculated for penicillin, ampicillin, amoxicillin, vancomycin, and erythromycin.

Antibacterial Peptide Susceptibility Assay

Gonococcal strains were grown to mid-log phase in GCBL with Kellogg's supplements and 0.042% $NaHCO_3$ and with 1 mM IPTG when appropriate, diluted 100-fold into GCBL medium without Kellogg's supplements and 0.042% $NaHCO_3$, and 90 µl aliquots of the diluted culture were mixed with 10 µl of the cationic antibacterial peptide LL-37 (provided by Dr. William Shafer, Emory University) and incubated in a 96-well polyproplylene microtiter plate. LL-37 (1 mg ml^{-1}) was serially diluted in twofold increments into 0.01% (v/v) acetic acid and 0.2% bovine serum albumin (E-media) [34] before being added to the diluted culture. Two microliters of diluted culture from each well and a positive control (gonococci without LL-37) were incubated for 1 h and then spotted onto GCB agar plates and incubated overnight at 37 °C, 5% CO_2 to determine the minimal growth inhibitory concentration (MGIC). All assays were performed twice in triplicate to confirm results. Data are reported as standard error of the mean (SEM) and the statistical significance was determined by a Student *t*-test.

RNA Isolation from Bacterial Cultures

MS11 and FA19 wild-type and their respective isogenic *ltgA* mutant stains, KC100 and LW9279, respectively, were grown under normal conditions and harvested at mid-log or stationary phase. Additionally, MS11 and KC100 were grown in the presence or absence of a sub-lethal concentration of penicillin (0.3 µg ml^{-1}) and harvested at mid-log phase. For growth experiments done in the presence or absence of N-acetyl-D-glucosamine (UDP-GlcNAc), MS11, KC100, and KH599 were grown to OD_{550} of 0.2, split into equal culture volumes, treated with and without 50 mM UDP-GlcNAc, and allowed to grow to mid-log phase before cells were harvested for RNA isolation. Total RNA from each experiment was isolated using the Ambion RiboPure-Bacteria kit (Applied Biosystems, Carlsbad, CA) following

the manufacturer's protocol. The quantity and quality of the RNA was determined by spectrophotometry and gel electrophoresis, respectively. RNA samples were checked for contaminating genomic DNA (gDNA) in a separate PCR reaction using ribonuclease B RNA (*rnpB*) primers (Table S1) but in the absence of reverse transcriptase; the *rnpB* primers were also used to amplify cDNA for the endogenous control in subsequent quantitative real-time PCR (qRT-PCR) experiments as described below.

Quantitative Real-Time PCR

Total RNA was reverse-transcribed to complementary DNA (cDNA) using the high-capacity cDNA Reverse-Transcription kit (Applied Biosystems, Carlsbad, CA) according to the manufacturer's protocol. Briefly, 1 µg of total RNA was mixed with 2 µl of 10X reverse transcription buffer, 0.8 µl 25X dNTPs (100 mM), 2 µl 10X random primers, 1 µl RNase inhibitor, and 1 µl MultiScribe reverse transcriptase (RT). Each reaction was incubated at room temperature for 10 min and then at 37 °C for 120 min before heat inactivation at 85 °C for 5 s. The resulting cDNA and gene-specific qRT-PCR primers (Table S1) were diluted to 300 ng and 5 µM, respectively, in a 25 µl reaction. Amplification was carried out in 96-well plates containing cDNA, qRT-PCR primers, and Sybr Green (Applied Biosystems, Carlsbad, CA). Differences in gene expression between mutant and wild-type were determined with the relative quantification method. All transcripts were assayed in triplicate on at least three or more independent biological samples with appropriate negative controls (minus RT to check for gDNA contamination) unless specified. The fold difference between mutant and wild-type for each transcript was determined by converting the differences in thresholds to fold change between strains based on the constitutively expressed *rnpB* gene (endogenous control). Thus, ΔCt values were normalized to *rnpB*.

Purification of Radioactively Labeled LOS and PG

MS11, the isogenic *ltgA* mutant strain in MS11 (KC100), and the complemented strain of KC100 (KH599) were grown to mid-log phase (OD_{550} of 0.6 to 0.8) in GCBL-medium containing Kellogg's supplements [33] and 0.042% (w/v) $NaHCO_3$ with aeration and then centrifuged for 5 min at 1700 × g. Cells were washed twice in 30 mls of pre-warmed GCBL containing 0.4% pyruvate in place of glucose as an energy source (GCBL-PMG) and then resuspended to an OD_{550} of 0.2 in 20 mls of GCBL-PMG. Cultures were allowed to grow for 2 h (OD_{550}, 0.6 to 0.8), at which time [6-^{3}H]glucosamine was added to each culture at final concentration of 2 µCi/ml. After a further 2 h of growth, aliquots from each culture were removed and used to determine the total amount of [6-^{3}H]glucosamine incorporated into cells and total protein levels. The remaining culture was split into two equal volumes, which were used to isolate LOS and PG.

Labeled LOS was purified using a modified procedure [35]. Briefly, pellets were washed in 1 ml of 1X PBS and then resuspended in 500 µl of 1X PBS. Fifty microliters of an LOS lysis buffer was added to 200 µl of the [6-^{3}H] glucosamine-labeled cells and the mixture was boiled for 10 min. Afterwards, samples were equilibrated to 60 °C for 15 min before adding 10 µl of 2.5 mg/ml Proteinase K and digesting for 1 h. For determination of overall radioactive incorporation, aliquots (2 µl) of the Proteinase K digest were removed and counted in a scintillation counter. The relative amounts of radioactivity incorporated into LOS in the different strains was determined by running the digests on a 16.5% Tricine-SDS-PAGE mini-gel at 105 V for 10 h, soaking the gel in EnHance, drying it on a gel dryer, and exposing the gel to film for 2–3 days. To purify PG, cells were resuspended in 4 ml of 50 mM sodium acetate (pH 5.0) and boiled for 30 min after adding 4 ml of 8% SDS. The boiled samples were ultracentrifuged at 25,000 rpm (Ti70 rotor) for 30 min. Then pellets were washed with 8 ml of molecular grade water and centrifuged for 30 min 4X before being resuspended in a final volume of 500 µl of water. The amount of [6-^{3}H] glucosamine-labeled PG for each strain was determined by counting 2 µl in a scintillation counter.

Results

Characterization of FA19 ΔltgA (LW9279)

In *Neisseria gonorrhoeae*, LtgA is one of the primary lytic transglycosylases responsible for release of peptidoglycan-derived cytotoxins [18]. Recently, it was reported that strains lacking *ltgA* showed a 50% reduction in the release of PG monomers and free disaccharides, with a concomitant increase in release of PG multimers, as well as a reduction in autolysis as cells entered stationary phase [19]. How *N. gonorrhoeae* responds at the cellular level to changes in PG release and recycling due loss of *ltgA* [18] is unclear. To address this question, we explored the effects on protein and gene transcription following deletion of *ltgA* under normal growth conditions in FA19. We insertionally inactivated *ltgA* (designated LW9279; Table 1) using a kanamycin-resistance cassette (*kanA*) and identified proteins whose expression was altered compared to the parental strain during exponential and stationary phase growth. We also examined the consequences of *ltgA* inactivation in MS11 (designated KC100; Table 1) because the effects of LtgA on PG recycling and release have been well-characterized in this strain. As previously reported for MS11 and KC100 [18], there were no differences in growth rate or colony morphology between

FA19 and LW9279 under normal growth conditions (data not shown).

LW9279 has an Altered Protein Expression Profile

We determined the effect of insertionally inactivating LtgA on protein expression by identifying protein spots (Table 2) and comparing the 2DGE protein profiles of strains FA19 (Fig. 1a) and LW9279 (Fig. 1b) during exponential and stationary phase growth. Comparative image analysis of the 2DGE gels revealed numerous proteins whose expression was either increased or decreased during exponential and stationary phase growth. Protein spots that were determined qualitatively and quantitatively to be altered in expression were selected and submitted to mass spectroscopy analysis. We analyzed 14 protein spots from the 2D gel and identified 8 proteins (Table S2). Mass spectrometry revealed that spot 1 (Fig. 1, top panels), which migrates close to the

Table 2 Proteins that were increased in LW9279 and their predicted functions

Spot	Identified protein	NCBI/ swissprot#	Gene	MW (kDa)	Predicted function
1	L-glutamine: D-fructose-6P amidotranferase[b]	Q9K1P9	*glmS*	66.4	Aminosugar metabolism
1a	*orf* NG0373 (putative glutamine transporter)[b]	15,793,954	NG0373	28.8	Amino acid transport
2	N-acetylglucosamine 1-phosphate uridyltransferase[b]	2,494,017	*glmU*	48.8	Aminosugar metabolism
3	Uracil phosphoribosyltransferase[a]	Q9K048	*uprT*	22.7	Nucleotide metabolism

Protein (s) isolated during mid-log ([a]) and stationary ([b]) to represent protein expression during stationary phase

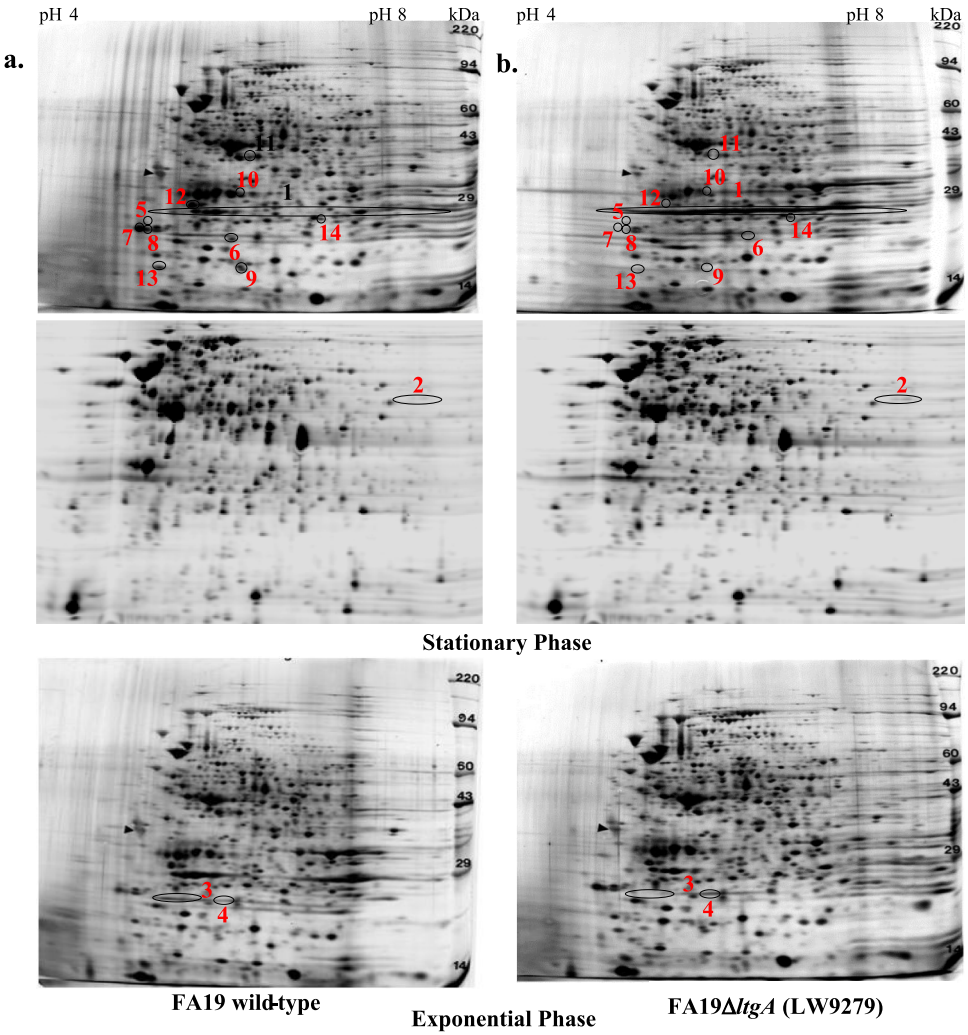

Fig. 1 Two-dimensional gel of proteins isolated from exponential and stationary phase gonococcal cultures. Panels are sections from the total protein profile image generated. Circled spots indicate proteins that were altered in expression between FAl9 wild-type (panel a) and LW9279 (panel b) during exponential and stationary phase. The circled spots were identified by mass spectrometry (Table 2 and S2)

29 kDa standard, produced peptide matches to two proteins, GlmS (66,400 Da; designated Protein 1 in Table 2), an L-glutamine:D-fructose-6-P amidotransferase [37], and open reading frame (*orf*) NG0373 (28,800 Da; designated Protein 1a in Table 2), a putative glutamine transporter [38]. Whereas the molecular mass of *orf* NG0373 is consistent with its mobility on 2DGE, the molecular mass of GlmS is much higher than the observed protein molecular mass on the 2D gel. This discrepancy was most likely due to proteolytic cleavage of GlmS during preparation of the proteins for 2DGE. Spot 2 (Fig. 1, middle panels) was identified as GlmU, a N-acetylglucosamine 1-phosphate uridyltransferase [39], while spot 3 (Fig. 1, lower panels) was identified as UprT, a uracil phosphoribosyltransferase [40]. GlmS, *orf* NG0373, and GlmU were identified from cells in stationary phase growth, while UprT was identified from cells in mid-log phase growth.

Intriguingly, the sequential enzymatic activities of these four proteins are involved in the synthesis of the key metabolic precursor, UDP-GlcNAc, which is needed for synthesis of LOS, PG, and other macromolecules. GlmS converts Fru-6-P to glucosamine-6-phosphate (GlcN-6-P) using glutamine (provided by *orf* NG0373) as an ammonia source. Once GlcN-6-P has been converted to GlcN-1-P (by GlmM), GlmU and UprT are responsible for the final conversion of GlcN-1-P to UDP-GlcNAc (Fig. 2).

LtgA Regulation of Cell Wall Synthesis Genes in *N. gonorrhoeae*

To ascertain if the increase in expression of these four proteins was due to changes in mRNA levels, we carried out qRT-PCR using gene-specific primers (Table S1) and total RNA extracted from exponential and stationary phase cultures of FA19 and LW9279. For comparison purposes, we also examined levels of these transcripts in strain MS11, KC100 (as MS11 but Δ*ltgA*) and KH599 (as KC100 but complemented with wild-type *ltgA*) (Table 1). In stationary cultures of LW9279, the levels of *glmS* and *glmU* transcripts were approximately fivefold higher than those in the parental strain FA19, while transcription of NG0373 was ~ threefold higher (Fig. 3a). In stationary cultures of KC100 (like LW9279, but in the MS11 background), RNA levels of *glmU, glmS,* and NG0373 were all increased to the same extent (~ fourfold) relative to MS11 (Fig. 3b). In the *ltgA*-complemented strain, KH599, addition of IPTG to induce expression of the complementing *ltgA* gene restored the mRNA levels of the three genes to near wild-type levels (Fig. 4a). These data suggest that the loss of *ltgA* is associated with increased levels of *orf* NG0373, *glmS*, and *glmU* transcripts. Although UprT was not detected by 2DGE in stationary phase cultures (Fig. 1), we decided to examine *uprT* transcript levels because of its role in UDP-GlcNAc production. qRT-PCR analysis indicated that the levels of *uprT* transcripts were approximately 2-fold higher in LW9279 and KC100 (Fig. 4b) during stationary phase compared to their respective parental strains but decreased to near wild-type levels in the complemented strain KH599 (Fig. 4a).

To enhance our understanding of how PG recycling and release affect UDP-GlcNAc gene expression, we examined the levels of *glmS* and *uprT* transcripts as representatives of the aminosugar and pyrimidine biosynthesis pathways in a

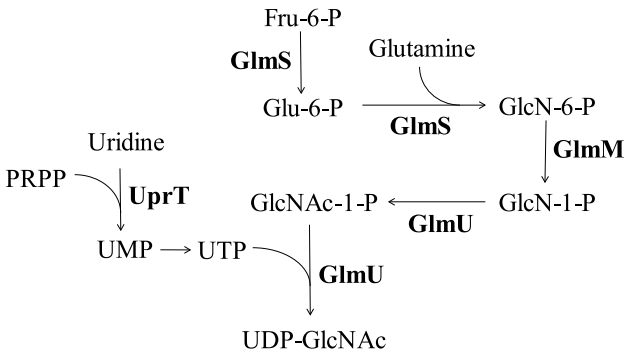

Fig. 2 Biosynthetic pathway for production of UDP-GlcNAc

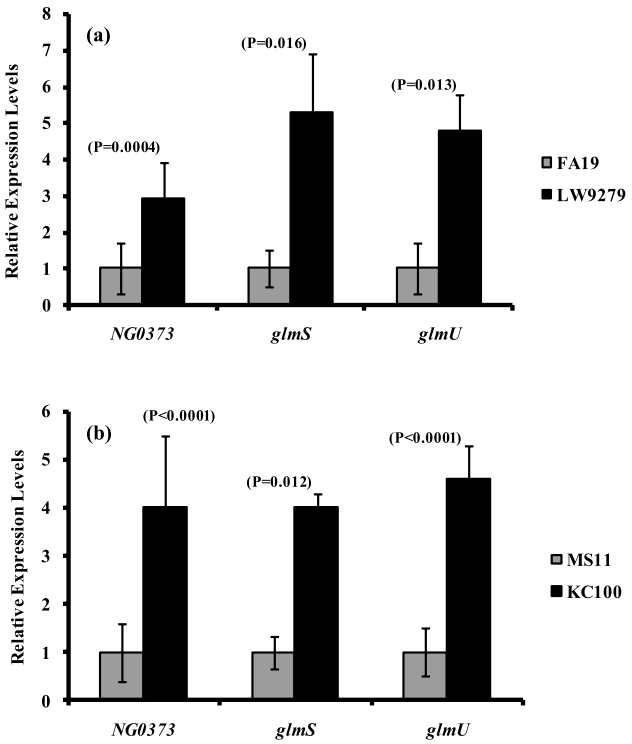

Fig. 3 Expression of *orf* NG0373, *glmS*, and *glmU* in strains, **a** LW9279 (FA19Δ*ltgA*) and **b** KC100 (MS11Δ*ltgA*), during stationary phase. Transcript levels were measured by qRT-PCR and indicate a change in expression levels in mutant relative to its respective parent strain. *P* values and SEM were determined from results of three or more independent experiments

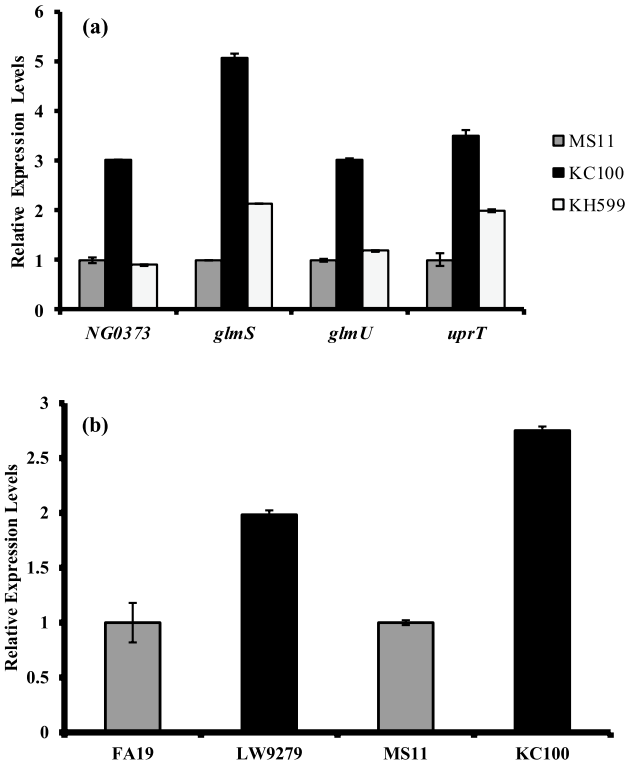

Fig. 4 **a** Expression of a putative ABC transporter (NG0373), *glmS*, *glmU,* and *uprT* during stationary phase in KC100 (MS11Δ*ltgA*) relative to MS11 wild-type. Orf NG0373 and *glmU* mRNA-transcript levels were restored back to wild-type level whereas *glmS* and *uprT* mRNA-transcript levels were restored back to a near wild-type level in strain KH599 (*ltgA* complement strain). A *P* value of < 0.0001 was determined and the SEM calculated from results of one representative experiment. **b** *uprT* expression during stationary phase in FA19 and MS11 wild-type strains and isogenic mutant *ltgA* strains (LW9279 and KC100, respectively). Transcript levels for *uprT* were measured by qRT-PCR and indicate the relative change in expression of *uprT* in mutant *ltgA* strains relative to wild-type. A *P* value of < 0.0001 was determined and SEM calculated from two independent experiments

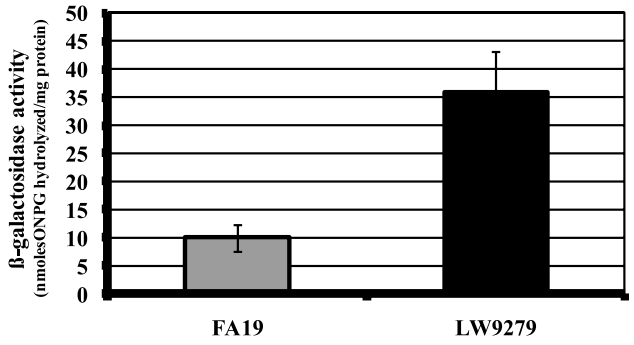

Fig. 5 Expression of *ponA* in a mutant *ltgA* strain. There was a 3.5-fold ($P = 0.0194$) increase in ß-galactosidase activity in LW9279 (FA19 Δ*ltgA*) relative to FA19 wildtype. The results are shown as the average values with a calculated stand error of the mean (SEM) determined from four independent assays

double-knock strain of *ltgA* and *ltgD* (designated KH560). Strain KH560 has significantly reduced PG recycling, increased PG multimer release, and a complete loss of PG monomer release [19]. These characteristics allowed us to determine if changes PG recycling affects genes involved in UDP-GlcNAc synthesis in *N. gonorrhoeae*. qRT-PCR experiments revealed 2- and 2.8-fold increases in *glmS* and *uprT* mRNA, respectively, in KH560 relative to MS11 (Fig. S1), which suggests that PG recycling also, may affect genes involved in UDP-GlcNAc synthesis.

Effect of UDP-GlcNAc on Aminosugar and Pyrimidine Gene Expression

mRNA-transcript levels of *orf* NG0373*, glmS*, *glmU*, and *uprT* are each markedly increased in gonococcal strains lacking *ltgA* (Figs. 3 and 4), potentially to maintain UDP-GlcNAc levels for synthesis of PG and LOS. A previous study showed that treatment of *S. aureus* with exogenous GlcNAc significantly decreased *glmS* promoter activity and protein levels compared to only a slight decrease in GlmS expression when treated with glucosamine (GlcN) [41]. Additionally, it has been reported GlmS is expendable in *E. coli* when exogenous GlcNAc is available [42]. This suggested that an exogenous GlcNAc may modulate expression of genes involved in de novo synthesis. To determine if the differences in expression of these genes in wild-type and mutant *ltgA* strains is due to a decrease in UDP-GlcNAc, mRNA levels of the four genes were determined in log phase cultures of MS11 and KC100 treated with exogenous UDP-GlcNAc or vehicle. The addition of 50 mM UDP-GlcNAc to KC100 decreased mRNA levels of NG0373, *glmS*, *glmU,* and *uprT* in mutant *ltgA* even below those in wild-type MS11 levels (Fig. 5) but had no obvious effect on growth (data not shown). Although, we do not know whether exogenous UDP-GlcNAc has a direct effect on UDP-GlcNAc synthesis genes, these results suggest that changes in PG recycling (due to inactivation of *ltgA*) [18] cause a shift from salvage of UDP-GlcNAc from recycled PG fragments to de novo synthesis in order to maintain levels of UDP-GlcNAc essential for PG and LOS biosynthesis.

Transcription of PBP 1 is Increased in an LtgA Mutant

Because loss of *ltgA* increases expression of genes involved in synthesis of UDP-GlcNAc, we investigated the effects of loss of *ltgA* on the expression of peptidoglycan synthesizing enzymes. Penicillin-binding protein 1 (PBP 1) encoded by the *ponA* gene is a bifunctional transglycosylase/transpeptidase that is responsible for the majority of new peptidoglycan added during cell growth, and its expression has been shown to be modulated by the MtrR transcriptional regulator

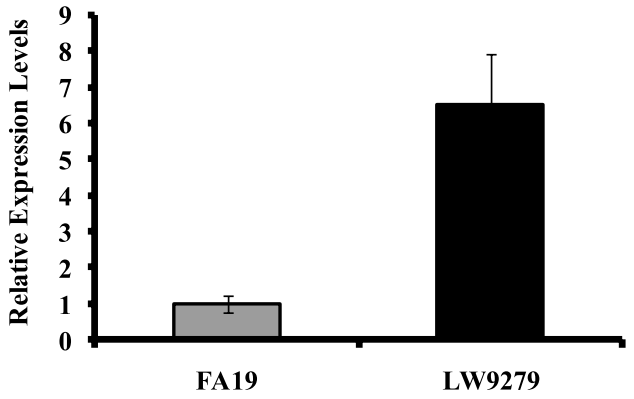

Fig. 6 Relative expression levels of *ponA* in mutant *ltgA*. qPCR results show that *ponA* mRNA-transcript levels were increased in strain LW9279 relative to FA19 wildtype. A *P* value of 0.04 indicates a significant difference in expression levels. The results are shown as the average values with a calculated stand error of the mean (SEM) determined from four independent assays

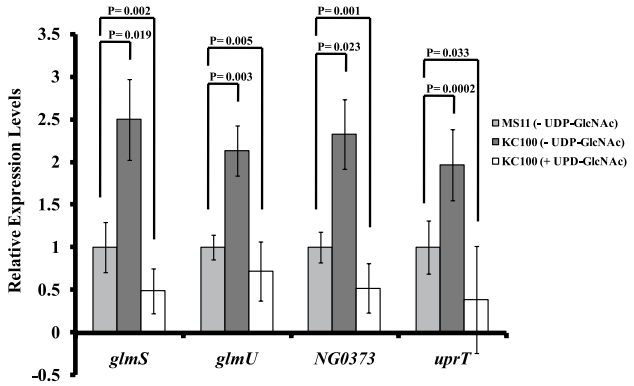

Fig. 7 The effect of N-acetyl-D-glucosamine (UDP-GlcNAc) on *orf* NG0373, *glmS*, *glmU,* and *uprT* mRNA-transcript levels. The addition of 50 mM UDP-GlcNAc to KC100 (+DP-GlcNAc) restored gene expression to a near wild-type levels. *P* values (located above bar) were determined-based replicates of three for each gene of at least two or more independent experiments

[43]. Interestingly, *ponA* shares an overlapping promoter that regulates a divergently transcribed operon encoding several pilus biosynthesis proteins, like PilO, which was identified as a protein spot altered in expression in strain LW9279 (Table S2 and Fig. 1). FA19 and LW9279 were transformed with constructs comprising the promoter and start codon of *ponA* fused to β-galactosidase [43] and the cultures transcriptional fusion grown in culture to mid-log phase. The cells were lysed and assessed for β-galactosidase activity (Fig. 6). As with the four genes involved in UDP-GlcNAc synthesis, the *ponA* gene was upregulated in the *ltgA* knockout strain relative to the parental. These results were also confirmed by isolating mRNA from FA19 and LW9279 and performing RT-PCR (Fig. 7).

Incorporation of GlcNAc into PG and LOS in Wild-Type and Mutant ltgA

Based on the capacity of exogenously added UDP-GlcNAc to restore the mRNA levels of enzymes involved in synthesis of UDP-GlcNAc to wild-type levels (Fig. 5), we next examined whether GlcNAc was used preferentially for PG or LOS synthesis in cells lacking *ltgA*. MS11 and KC100 (MS11 Δ*ltgA)* were metabolically labeled with [6-^{3}H]glucosamine in the presence of pyruvate as a carbon source (the change in carbon source did not affect *glmS* mRNA transcription; data not shown). After two hours of labeling, equivalent amounts of cultures were used to purify LOS and PG to determine if there was a difference in the amount of [6-^{3}H] glucosamine incorporated during synthesis between wild-type and mutant *ltgA*. There was no difference in [6-^{3}H]glucosamine incorporation into LOS or PG (Fig. S2) between MS11 and KC100 on a per weight basis, suggesting that the levels of the UDP-GlcNAc pool in both MS11 and KC100 are very similar, with the increased expression of *orf* NG0373*, glmS*, *glmU*, and *uprT* in KC100 collaborating to maintain normal levels of the UDP-GlcNAc pool to ensure that an adequate precursor supply is available for both LOS and PG synthesis.

Penicillin Treatment Increases Expression of Aminosugar and Pyrimidine Biosynthesis Genes

While we have shown that the loss of *ltgA* leads to increased mRNA-transcript levels of genes responsible for production of UDP-GlcNAc (Fig. 3), it remained unclear whether changes in mRNA levels were an adaptive response as a result of changes in PG recycling due to loss of *ltgA* [18]. Sinha et al. [44] showed that treatment of *N. gonorrhoeae* RD_5 with penicillin G accelerated PG turnover and concomitant release of [^{3}H]glucosamine-containing fragments. Therefore, we determined whether treatment of MS11 and KC100 with a sub-lethal concentration of penicillin G (0.3 μg/ml) would produce an adaptive response in *N. gonorrhoeae* and whether such a response would be additive with that induced by loss of *ltgA*.

The levels of *glmS* and *uprT* mRNA were quantified in MS11 cells grown for 1 h in the presence or absence of a sub-lethal concentration of penicillin. mRNA transcripts of *glmS* and *uprT* increased 3.6- and fourfold, respectively, in penicillin-treated cells relative to untreated MS11 cells (Fig. 8a, b), which was like the adaptive response observed in KC100 without penicillin (Fig. 8a, b). To determine if the effects of penicillin on mRNA transcription were additive with those due to loss of LtgA, we quantified the levels of *glmS* and *uprT* mRNA transcripts by growing KC100 for 1 h in the presence or absence of the same sub-lethal concentration of penicillin as above. Levels of *glmS* and *uprT* mRNA increased 9- and sevenfold, respectively, in penicillin-treated

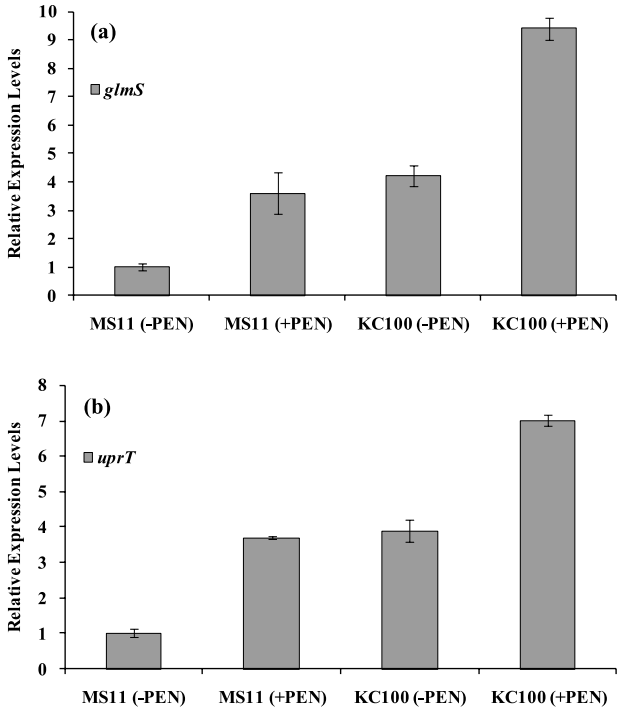

Fig. 8 Relative expression levels of (**a**). *glmS* and (**b**). *uprT* for penicillin (+PEN) treated strains relative to MS11-untreated (−PEN). Transcript levels were measured by qRT-PCR and indicate the relative change in gene expression between strains. A *P* value of 0.014 and 0.001 was calculated for *glmS* and *uprT*, respectively. Values and the SEM were calculated from the results of three independent experiments

KC100, which was twofold higher than that of penicillin-treated MS11 and untreated KC100 (Fig. 8a, b). These data indicate that the gonococcus undergoes adaptive responses both to changes in cell wall remodeling (i.e., from loss of LtgA) and to inhibition of normal PG synthesis (i.e., from sub-lethal penicillin treatment) by increasing expression of genes responsible for UDP-GlcNAc synthesis, and the resulting increases in transcription of *glmS* and *uprT* in the two conditions are additive.

Mutant ltgA Strains Show Increased Sensitivity to Antimicrobials

Mutants of murein hydrolases have been reported to increase susceptibility to antimicrobials [27, 45]; therefore, we determined if loss of LtgA increases susceptibility to antimicrobials in MS11, FA19, and their respective isogenic *ltgA* mutant strains. KC100 was 2- to 3-fold more susceptible to β-lactam antibiotics compared to the parental MS11 strain, whereas the differences between LW9279 and FA19 were less obvious (Table 3). Moreover, the complemented strain KH599 showed β-lactam susceptibilities similar to wild-type, but only in the presence of IPTG to induce expression of LtgA. Unexpectedly, both mutant *ltgA* strains (LW9279 and KC100) showed an increased sensitivity to vancomycin, an antibiotic normally ineffective against Gram-negative bacteria due to its inability to cross the outer membrane barrier; thus, the *ltgA* strains appear to be more permeable than the parental strains. There were no significant differences in susceptibility to erythromycin among the tested strains. FA19 and MS11 wild-type strains were ~2-fold less susceptible to LL-37 compared to isogenic LW9279 and KC100, respectively (Table 3). Studies in other Gram-negative bacteria have shown that mutations in multiple hydrolases are necessary for a higher degree of antimicrobial susceptibility [27, 45], but for *N. gonorrhoeae*, altered susceptibility was observed following deletion of only LtgA.

The increased susceptibility of KC100 to LL-37 and vancomycin suggests that loss of LtgA alters the cell envelope to make it more permeable, perhaps by altering its structure.

Table 3 Susceptibility of gonococcal stains to antimicrobials

Strain	Description	Minimum growth inhibitory concentrations (MGIC, μg ml^{-1})					
		PEN[a]	AMP[b]	AMX[c]	ERY[d]	VAN[e]	LL-37[f]
FA19	Wild type	>0.016	0.023	0.032	0.25	8	7.25(±1.05)
LW9279	FA19 *ltgA:Kan*^{R}	>0.016	0.023	0.016	0.25	4	3.1
MS11	Wild type	1	1	1	2	24	1.5
KC100	MS11 *ltgA* deletion	0.5	0.5	0.38	2	12	.78
KH599[g]	KC100 complement	1	ND	0.75	2	24	ND
KH599[h]	KC100 complement	ND	ND	0.38	ND	ND	ND

PEN[a], penicillin; AMP[b], ampicillin; AMX[c], amoxicillin; ERY[d], erythromycin; VAN[e], vancomycin; LL-37[f], human-cathelicidin

ND, not done

*$P<0.0001$, *P* values were determined using Two-way ANOVA. Assays were done in triplicate two or more times with a standard deviation (SD) equal to ±0.000 unless otherwise reported

[g]with IPTG

[h]without IPTG

Therefore, we determined whether there was a difference in membrane permeability due to changes in LOS. Examination of LOS from MS11 and KC100 showed that the two preparations were indistinguishable with respect to LOS amount and its migration rate and migration distance on SDS-PAGE (Fig. S3).

Discussion

The aim of this study was to identify changes in protein and gene expression in Neisseria gonorrhoeae due to loss of *ltgA*. We observed a coordinated increase in protein and mRNA transcripts for four genes involved in UDP-GlcNAc synthesis (orf NG0373, *glmS*, *uprT*, and *glmU*) under normal growth conditions and exposure to sub-lethal concentrations of penicillin in two distinct strains of *N. gonorrhoeae* lacking LtgA. This study is the first to examine the effects of PG turnover and antibiotic-induced PG degradation in *N. gonorrhoeae* on the synthesis of UDP-GlcNAc, a precursor for both PG and lipooligosaccharide (LOS) synthesis.

While our data showed changes in transcription of UDP-GlcNAc synthesis genes due to loss of *ltgA*, it remains unclear whether the increase is in direct response to changes in PG recycling and release, or to a general stress response to a possible defect in cell wall assembly. It is plausible that the loss of LtgA activity may lead to decreased PG fragment recycling and subsequent reduction of intracellular pools of salvaged precursors, directly impacting transcription of genes responsible for UDP-GlcNAc synthesis through the action of an unknown regulator. Recycled PG fragments have been shown to regulate gene expression in other Gram-negative bacteria [46]. Alternatively, the increase in mRNA-transcripts of enzymes involved in UDP-GlcNAc synthesis in mutant *ltgA* strains could reflect the capacity of *N. gonorrhoeae* to detect disruption of its cell wall. Loss of LtgA could exert stress by incomplete removal of older PG due to reduced PG turnover in the *ltgA* mutant strain, which might produce gonococci with weakened cell walls and cause cells to be more susceptible to environmental changes during growth [47]. Also, this may explain why *ltgA* mutants are more susceptible to antimicrobials. Interestingly, the expression of *glmS* was significantly increased in *ltgA* mutants and reduced by exogenous UDP-GlcNAc, which was also shown to be modulated by PG components in other bacteria [41]. This suggests a physiological reason why the cell would need to modulate GlmS activity and why a delicate balance must be maintained between UDP-GlcNAc de novo synthesis and PG recycling [48].

Studies have shown that some bacteria respond to changes in cell wall integrity and/or altered levels of intracellular PG fragments [46]. Our findings suggest that, like deletion of *ltgA*, a sub-lethal amount of penicillin G added during log phase growth may decrease intracellular pools of UDP-GlcNAc by decreasing recycling, which increases expression of genes involved in UDP-GlcNAc synthesis. Alternatively, gonococcus may be capable of sensing changes in the cross-linking of cell wall peptides when treated with penicillin, which may be important for maintaining a balance between PG synthesis and degradation or sensing environmental changes that might be detrimental during growth. The mechanisms by which most bacteria monitor intracellular levels of peptidoglycan (PG) precursors remain largely unknown. Recent findings indicate that transcriptional regulation in *N. gonorrhoeae* responds to amino sugar levels [49]. The phenotypes of PG recycling mutants suggest that *N. gonorrhoeae* can regulate sugar metabolism based on intracellular levels of PG fragments. For instance, an *ampD* mutant, unable to degrade PG fragments beyond the anhydro-MurNAc-tripeptide, showed increased uptake and metabolism of free disaccharides [28, 31, 50]. Similarly, *ltgA ltgD* double mutants, which do not produce anhydro-PG monomers, the major substrate for PG recycling, also consume free disaccharides, indicating upregulated uptake and degradation factors for PG-derived sugars [19]. Unlike *Escherichia coli* and many other bacterial species, *Neisseria gonorrhoeae* lacks a homolog of AmpR [15, 27, 31], making it unclear if they can regulate transcription based on the levels of recycled PG fragments or PG biosynthetic precursors. However, in this study, findings suggest that transcriptional regulation in *N. gonorrhoeae* responds to amino sugar levels and/or changes in the cell envelope. The phenotypes of PG recycling mutants suggest that *N. gonorrhoeae* can regulate sugar metabolism based on intracellular levels of PG fragments. In *Escherichia coli*, deletion of several lytic transglycosylases resulted in increased sensitivity to antibiotics that are typically ineffective against gram-negative bacteria. In contrast, the single LT deletion (*ltgA*) in *N. gonorrhoeae* affects its sensitivity to antibiotics and antibacterial peptides. The increased sensitivity to the β-lactam antibiotics may be related to a delicate balance between cell wall degradation by LTs and synthesis by penicillin-binding proteins during growth. These findings underscore the complex interplay between PG recycling, cell wall synthesis, and antibiotic resistance in bacteria, emphasizing the importance of understanding these processes for developing effective antimicrobial strategies.

Conclusion

The findings of this study highlight a significant adaptive mechanism, where the increase in mRNA-transcripts for UDP-GlcNAc synthesis genes in mutant *ltgA* serves as a response to cell wall stress and/or penicillin-induced treatment. Induced changes in mRNA-transcript levels for

UDP-GlcNAc synthesis genes highlights a potential link between cell wall stress and antibiotic resistance mechanisms. This adaptive response ensures the maintenance of adequate UDP-GlcNAc levels necessary for lipooligosaccharide (LOS) and peptidoglycan (PG) synthesis. By favoring the salvage pathway for UDP-GlcNAc synthesis, bacteria may be able to circumvent the inhibitory effects of antibiotics on cell wall biosynthesis, thereby contributing to their survival and resistance. Additionally, a decrease in cytoplasmic pools of UDP-GlcNAc may be the primary driver of this transcriptional change, as evidenced by the restoration of gene transcript levels upon the addition of UDP-GlcNAc. The ability of bacteria to adapt their metabolic pathways in response to due loss of *ltgA* and/or antibiotic-induced stress suggests that targeting these adaptive mechanisms could be a viable strategy for developing new antimicrobial therapies. Understanding the interplay between metabolic adaptation and antibiotic resistance can inform the design of drugs that disrupt these adaptive responses, potentially enhancing the efficacy of existing antibiotics and reducing the emergence of resistant strains.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00284-025-04261-7.

Acknowledgements We would like to thank Dr. Joseph P. Dillard from the University of Wisconsin-Madison for providing MS11 strains and Dr. William Shafer from Emory University for providing certain antibacterial peptides and experimental suggestions.

Author Contributions All authors contributed to the experimental design and conception of the manuscript. Experimental execution, data collection, and analysis were completed by Candra O. Broadie and Alaa I. Telchy. Dr. Joseph P. Dillard, Dr. Antionio T. Baines, and Dr. Robert A. Nicholas contributed to manuscript's intellectual content. Candra O. Broadie was responsible for the initial draft of the manuscript while Dr. Daniel Williams was responsible for final review and giving approval for publication. All authors read and approved the final manuscript.

Funding This work was supported by National Institutes of Health (5SC2 A1081656-02 to Daniel Williams).

Data Availability All data in the current study and supporting materials are included in this published article and are available from the corresponding author upon reasonable request.

Code Availability Not applicable.

Declarations

Conflicts of interests The authors declare there are not conflicts of interests in these areas.

Ethical Approval Not applicable.

Consent to Participate Not required.

Consent to Publications Not required.

References

1. Chesson HW, Kent CK, Owusu-Edusei K Jr, Leichliter JS, Aral SO (2012) Disparities in sexually transmitted disease rates across the "Eight Americas." Sex Transm Dis 39(6):458–464. https://doi.org/10.1097/OLQ.0b013e318248e3eb
2. Azariah S, Perkins N (2007) Risk factors and characteristics of patients with gonorrhoea presenting to Auckland sexual health service, New Zealand. N Z Med J 120(1252):U2491
3. Hobbs MM, Sparling PF, Cohen MS, Shafer WM, Deal CD, Jerse AE (2011) Experimental gonococcal infection in Male volunteers: cumulative experience with *Neisseria gonorrhoeae* strains FA1090 and MS11mkC. Front Microbiol 2:123. https://doi.org/10.3389/fmicb.2011.00123
4. Seifert HS, Wright CJ, Jerse AE, Cohen MS, Cannon JG (1994) Multiple gonococcal pilin antigenic variants are produced during experimental human infections. J Clin Invest 93(6):2744–2749. https://doi.org/10.1172/JCI117290
5. Wasserheit JN (1992) Epidemiological synergy. interrelationships between human immunodeficiency virus infection and other sexually transmitted diseases. Sex Transm Dis 19:61–77
6. Unemo M (2015) Current and future antimicrobial treatment of gonorrhoea—the rapidly evolving Neisseria gonorrhoeae continues to challenge. BMC Infect Dis 15:364. https://doi.org/10.1186/s12879-015-1029-2
7. Unemo M, Nicholas RA (2012) Emergence of multidrug-resistant, extensively drug-resistant and untreatable gonorrhea. Future Microbiol 7(12):1401–1422. https://doi.org/10.2217/fmb.12.117
8. CDC (2012) Update to CDC's sexually transmitted diseases treatment guidelines, 2010: oral cephalosporins no longer a recommended treatment for gonococcal infections. MMWR Morb Mortal Wkly Rep 61:590–4
9. Tapsall JW (2009) Implications of current recommendations for third-generation cephalosporin use in the WHO western Pacific region following the emergence of multiresistant gonococci. Sex Transm Infect 85(4):256–258. https://doi.org/10.1136/sti.2008.035337
10. Fleming TJ, Wallsmith DE, Rosenthal RS (1986) Arthropathic properties of gonococcal peptidoglycan fragments: implications for the pathogenesis of disseminated gonococcal disease. Infect Immun 52(2):600–608. https://doi.org/10.1128/iai.52.2.600-608.1986
11. Melly MA, McGee ZA, Rosenthal RS (1984) Ability of monomeric peptidoglycan fragments from Neisseria gonorrhoeae to damage human fallopian-tube mucosa. J Infect Dis 149(3):378–386. https://doi.org/10.1093/infdis/149.3.378

12. Scheurwater E, Reid CW, Clarke AJ (2008) Lytic transglycosylases: bacterial space-making autolysins. Int J Biochem Cell Biol 40(4):586–591. https://doi.org/10.1016/j.biocel.2007.03.018
13. Romeis T, Holtje JV (1994) Specific interaction of penicillin-binding proteins 3 and 7/8 with soluble lytic transglycosylase in Escherichia coli. J Biol Chem 269(34):21603–21607
14. Vollmer W, von Rechenberg M, Holtje JV (1999) Demonstration of molecular interactions between the murein polymerase PBP1B, the lytic transglycosylase MltA, and the scaffolding protein MipA of *Escherichia coli*. J Biol Chem 274(10):6726–6734. https://doi.org/10.1074/jbc.274.10.6726
15. Jacobs C, Frere JM, Normark S (1997) Cytosolic intermediates for cell wall biosynthesis and degradation control inducible beta-lactam resistance in gram-negative bacteria. Cell 88(6):823–832. https://doi.org/10.1016/s0092-8674(00)81928-5
16. Wong W, Chatterjee AN, Young FE (1978) Regulation of bacterial cell walls: correlation between autolytic activity and cell wall turnover in *Staphylococcus aureus*. J Bacteriol 134(2):555–561. https://doi.org/10.1128/jb.134.2.555-561.1978
17. Holtje JV (1996) Lytic transglycosylases. EXS 75:425–429. https://doi.org/10.1007/978-3-0348-9225-4_21
18. Cloud KA, Dillard JP (2002) A lytic transglycosylase of *Neisseria gonorrhoeae* is involved in peptidoglycan-derived cytotoxin production. Infect Immun 70(6):2752–2757. https://doi.org/10.1128/IAI.70.6.2752-2757.2002
19. Cloud-Hansen KA, Hackett KT, Garcia DL, Dillard JP (2008) *Neisseria gonorrhoeae* uses two lytic transglycosylases to produce cytotoxic peptidoglycan monomers. J Bacteriol 190(17):5989–5994. https://doi.org/10.1128/JB.00506-08
20. Cloud KA, Dillard JP (2004) Mutation of a single lytic transglycosylase causes aberrant septation and inhibits cell separation of *Neisseria gonorrhoeae*. J Bacteriol 186(22):7811–7814. https://doi.org/10.1128/JB.186.22.7811-7814.2004
21. Dillard JP, Seifert HS (2001) A variable genetic island specific for *Neisseria gonorrhoeae* is involved in providing DNA for natural transformation and is found more often in disseminated infection isolates. Mol Microbiol 41(1):263–277. https://doi.org/10.1046/j.1365-2958.2001.02520.x
22. Kohler PL, Hamilton HL, Cloud-Hansen K, Dillard JP (2007) AtlA functions as a peptidoglycan lytic transglycosylase in the *Neisseria gonorrhoeae* type IV secretion system. J Bacteriol 189(15):5421–5428. https://doi.org/10.1128/JB.00531-07
23. Flak TA, Goldman WE (1998) Muramyl peptide probes derived from tracheal cytotoxin of *Bordetella pertussis*. Anal Biochem 264(1):41–46
24. Dokter WH, Dijkstra AJ, Koopmans SB, Stulp BK, Keck W, Halie MR et al (1994) G(Anh)MTetra, a natural bacterial cell wall breakdown product, induces interleukin-1 beta and interleukin-6 expression in human monocytes. A study of the molecular mechanisms involved in inflammatory cytokine expression. J Biol Chem. 269:4201–6
25. Heiss LN, Moser SA, Unanue ER, Goldman WE (1993) Interleukin-1 is linked to the respiratory epithelial cytopathology of pertussis. Infect Immun 61(8):3123–3128. https://doi.org/10.1128/iai.61.8.3123-3128.1993
26. Nigro G, Fazio LL, Martino MC, Rossi G, Tattoli I, Liparoti V et al (2008) Muramylpeptide shedding modulates cell sensing of Shigella flexneri. Cell Microbiol 10(3):682–695
27. Korsak D, Liebscher S, Vollmer W (2005) Susceptibility to antibiotics and beta-lactamase induction in murein hydrolase mutants of *Escherichia coli*. Antimicrob Agents Chemother 49(4):1404–1409. https://doi.org/10.1128/AAC.49.4.1404-1409.2005
28. Nunez C, Moreno S, Cardenas L, Soberon-Chavez G, Espin G (2000) Inactivation of the ampDE operon increases transcription of algD and affects morphology and encystment of *Azotobacter vinelandii*. J Bacteriol 182(17):4829–4835. https://doi.org/10.1128/JB.182.17.4829-4835.2000
29. Chahboune A, Decaffmeyer M, Brasseur R, Joris B (2005) Membrane topology of the *Escherichia coli* AmpG permease required for recycling of cell wall anhydromuropeptides and AmpC beta-lactamase induction. Antimicrob Agents Chemother 49(3):1145–1149. https://doi.org/10.1128/AAC.49.3.1145-1149.2005
30. Mengin-Lecreulx D, Siegel E, van Heijenoort J (1989) Variations in UDP-N-acetylglucosamine and UDP-N-acetylmuramyl-pentapeptide pools in *Escherichia coli* after inhibition of protein synthesis. J Bacteriol 171(6):3282–3287. https://doi.org/10.1128/jb.171.6.3282-3287.1989
31. Jacobs C, Huang LJ, Bartowsky E, Normark S, Park JT (1994) Bacterial cell wall recycling provides cytosolic muropeptides as effectors for beta-lactamase induction. EMBO J 13(19):4684–4694. https://doi.org/10.1002/j.1460-2075.1994.tb06792.x
32. Ramos-Aires J, Plesiat P, Kocjancic-Curty L, Kohler T (2004) Selection of an antibiotic-hypersusceptible mutant of *Pseudomonas aeruginosa*: identification of the GlmR transcriptional regulator. Antimicrob Agents Chemother 48(3):843–851. https://doi.org/10.1128/AAC.48.3.843-851.2004
33. Kellogg DS Jr, Peacock WL Jr, Deacon WE, Brown L, Pirkle DI (1963) *Neisseria Gonorrhoeae*. I. virulence genetically linked to clonal variation. J Bacteriol 85:1274–9. https://doi.org/10.1128/jb.85.6.1274-1279.1963
34. Shafer WM, Qu X, Waring AJ, Lehrer RI (1998) Modulation of *Neisseria gonorrhoeae* susceptibility to vertebrate antibacterial peptides due to a member of the resistance/nodulation/division efflux pump family. Proc Natl Acad Sci U S A 95(4):1829–1833
35. Schagger H, von Jagow G (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal Biochem 166(2):368–379. https://doi.org/10.1016/0003-2697(87)90587-2
36. Maness MJ, Sparling PF (1973) Multiple antibiotic resistance due to a single mutation in *Neisseria gonorrhoeae*. J Infect Dis 128(3):321–330. https://doi.org/10.1093/infdis/128.3.321
37. Milewski S (2002) Glucosamine-6-phosphate synthase–the multi-facets enzyme. Biochim Biophys Acta 1597(2):173–192. https://doi.org/10.1016/s0167-4838(02)00318-7
38. Nohno T, Saito T, Hong JS (1986) Cloning and complete nucleotide sequence of the *Escherichia coli* glutamine permease operon (glnHPQ). Mol Gen Genet 205(2):260–269. https://doi.org/10.1007/BF00430437
39. Ullrich J, van Putten JP (1995) Identification of the gonococcal glmU gene encoding the enzyme N-acetylglucosamine 1-phosphate uridyltransferase involved in the synthesis of UDP-GlcNAc. J Bacteriol 177(23):6902–6909. https://doi.org/10.1128/jb.177.23.6902-6909.1995
40. Arsene-Ploetze F, Nicoloff H, Kammerer B, Martinussen J, Bringel F (2006) Uracil salvage pathway in *Lactobacillus plantarum*: Transcription and genetic studies. J Bacteriol 188(13):4777–4786. https://doi.org/10.1128/JB.00195-06
41. Komatsuzawa H, Fujiwara T, Nishi H, Yamada S, Ohara M, McCallum N et al (2004) The gate controlling cell wall synthesis in *Staphylococcus aureus*. Mol Microbiol 53(4):1221–1231. https://doi.org/10.1111/j.1365-2958.2004.04200.x
42. Kalamorz F, Reichenbach B, Marz W, Rak B, Gorke B (2007) Feedback control of glucosamine-6-phosphate synthase GlmS expression depends on the small RNA GlmZ and involves the novel protein YhbJ in *Escherichia coli*. Mol Microbiol 65(6):1518–1533. https://doi.org/10.1111/j.1365-2958.2007.05888.x
43. Folster JP, Dhulipala V, Nicholas RA, Shafer WM (2007) Differential regulation of ponA and pilMNOPQ expression by the MtrR transcriptional regulatory protein in Neisseria gonorrhoeae.

J Bacteriol 189(13):4569–4577. https://doi.org/10.1128/JB.00286-07
44. Sinha RK, Rosenthal RS (1981) Effect of penicillin G on release of peptidoglycan fragments by Neisseria gonorrhoeae: characterization of extracellular products. Antimicrob Agents Chemother 20(1):98–103. https://doi.org/10.1128/AAC.20.1.98
45. Heidrich C, Ursinus A, Berger J, Schwarz H, Holtje JV (2002) Effects of multiple deletions of murein hydrolases on viability, septum cleavage, and sensitivity to large toxic molecules in *Escherichia coli*. J Bacteriol 184(22):6093–6099. https://doi.org/10.1128/JB.184.22.6093-6099.2002
46. Borisova M, Gaupp R, Duckworth A, Schneider A, Dalugge D, Muhleck M et al (2016) Peptidoglycan recycling in gram-positive bacteria is crucial for survival in stationary phase. mBio. https://doi.org/10.1128/mBio.00923-16
47. Jordan S, Hutchings MI, Mascher T (2008) Cell envelope stress response in gram-positive bacteria. FEMS Microbiol Rev 32(1):107–146. https://doi.org/10.1111/j.1574-6976.2007.00091.x
48. Patel V, Wu Q, Chandrangsu P, Helmann JD (2018) A metabolic checkpoint protein GlmR is important for diverting carbon into peptidoglycan biosynthesis in Bacillus subtilis. PLoS Genet. https://doi.org/10.1371/journal.pgen.1007689
49. Ayala JC, Balthazar JT, Shafer WM (2024) Transcriptional responses of Neisseria gonorrhoeae to glucose and lactate: implications for resistance to oxidative damage and biofilm formation. mBio. https://doi.org/10.1128/mbio.01761-24
50. Garcia DL, Dillard JP (2008) Mutations in ampG or ampD affect peptidoglycan fragment release from Neisseria gonorrhoeae. J Bacteriol 190(11):3799–3807. https://doi.org/10.1128/JB.01194-07