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Neisseria gonorrhoeae Uses Two Lytic Transglycosylases To Produce Cytotoxic Peptidoglycan Monomers▽†

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Peptidoglycan fragments released by *Neisseria gonorrhoeae* contribute to the inflammation and ciliated cell death associated with gonorrhea and pelvic inflammatory disease. However, little is known about the production and release of these fragments during bacterial growth. Previous studies demonstrated that one lytic transglycosylase, LtgA, was responsible for the production of approximately half of the released peptidoglycan monomers. Systematic mutational analysis of other putative lytic transglycosylase genes identified lytic transglycosylase D (LtgD) as responsible for release of peptidoglycan monomers from gonococci. An *ltgA ltgD* double mutant was found not to release peptidoglycan monomers and instead released large, soluble peptidoglycan fragments. In pulse-chase experiments, recycled peptidoglycan was not found in cytoplasmic extracts from the *ltgA ltgD* mutant as it was for the wild-type strain, indicating that generation of anhydro peptidoglycan monomers by lytic transglycosylases facilitates peptidoglycan recycling. The *ltgA ltgD* double mutant showed no growth abnormalities or cell separation defects, suggesting that these enzymes are involved in pathogenesis but not necessary for normal growth.

Peptidoglycan (PG) fragments released during growth contribute to the pathogenesis of multiple bacterial infections, including those of *Bordetella pertussis*, *Helicobacter pylori*, and *Neisseria gonorrhoeae* (6, 23, 34). PG fragments induce the production of inflammatory cytokines, cause ciliated cell damage and fluid efflux, and trigger the Nod signaling cascade (reviewed in reference 5). Although PG fragments have been studied biochemically and for immunologic effects in multiple systems, the repertoire of genes and enzymes involved in PG fragment production and release from growing bacteria is unknown.

PG fragments released from gram-negative bacterial pathogens are predicted to be produced by the action of lytic transglycosylases. Lytic transglycosylases cleave the *N*-acetylmuramic acid–β-1,4-*N*-acetylglucosamine linkage in PG and catalyze the formation of a 1,6-anhydro bond on the *N*-acetylmuramic acid (16). PG monomers released from *N. gonorrhoeae* and *B. pertussis* were shown to have the 1,6-anhydro bond, indicating that they were generated by lytic transglycosylases (26, 30). To identify genes for PG monomer production, we systematically mutated the genes for lytic transglycosylase homologues in *N. gonorrhoeae*. Mutation of lytic transglycosylase A (*ltgA*) resulted in a substantial decrease in PG monomers released (3). Mutations in lytic transglycosylase B (*ltgB*) or lytic transglycosylase C (*ltgC*) genes had no effect on PG monomer release (4, 19), although the *ltgC* mutant showed a severe defect in cell separation. These findings suggested the presence of other lytic transglycosylases in *N. gonorrhoeae* involved in release of PG monomers.

Here we show that lytic transglycosylase LtgD is involved in the release of PG monomers. Additionally, we generated and characterized an *N. gonorrhoeae* strain deleted for both *ltgA* and *ltgD* and found that this strain does not release PG monomers. Our studies demonstrate that lytic transglycosylases in *N. gonorrhoeae* have specific functions and that LtgA and LtgD are responsible for the production of 1,6-anhydro PG monomers, a virulence factor in gonococcal infections.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All strains and plasmids used in this study are listed in Table 1. All gonococcal strains are derivatives of MS11 (31), and all experiments except transformations were performed with nonpiliated variants. Gonococci were grown with aeration in GC base liquid (GCBL) medium (1.5% proteose peptone no. 3, 0.4% K_2HPO_4, 0.1% KH_2PO_4, 0.1% NaCl; pH 7.2) containing Kellogg's supplements and 0.042% $NaHCO_3$ or on GCB agar plates (Difco) in the presence of 5% CO_2 at 37°C (18, 24). *Escherichia coli* was grown in Luria broth or on Luria agar plates (27). Antibiotics were used at the following concentrations: for *N. gonorrhoeae*, 10 μg of erythromycin (Erm) per ml and 100 μg of streptomycin (Str) per ml; for *E. coli*, 500 μg of Erm per ml, 40 μg of kanamycin (Kan) per ml, and 100 μg of Str per ml.

Growth rate comparisons. To evaluate the effects of the *ltgA* and *ltgD* mutations on growth, the *ltgA ltgD* double mutant KH560 and its wild-type parent strain MS11 were grown in liquid culture in either complex medium (GCBL) as described above or in defined medium (Graver-Wade medium) (35). *N. gonorrhoeae* strains were grown on GCB agar plates for 20 h and then the bacteria were inoculated into the liquid medium at a density of approximately 10^8 CFU/ml (optical density at 540 nm [OD_{540}], 0.2). The cultures were grown at 37°C with aeration, and optical density readings (OD_{540}) were taken at 0, 1, 2, 4, 6, 8, 10, 12, and 24 h postinoculation. The numbers of viable bacteria were also determined at 10 h postinoculation by plating serial dilutions of the cultures on GCB agar plates.

Plasmid construction. For cloning of *ltgD*, the following specific primers were designed based on the sequence of *N. gonorrhoeae* strain FA1090 (GenBank accession no. AE004969): 5′-AAACCCTCGCCACGGAATACACTT-3′ and 5′-CCCTTCAATCCCTTGCTGCGTAAAA-3′. *ltgD* was amplified from MS11 chromosomal DNA using an annealing temperature of 60°C. The *ltgD* PCR product was digested with EcoRV and BclI and ligated into the BamHI and EcoRV sites of pKC1 (3), forming pKC12. An internal deletion in *ltgD* was formed by digesting pKC12 with SmaI, removing 1 kb of coding sequence (pKC14). The *ltgD* deletion region from pKC14 was excised by digestion with HindIII and SpeI and then subcloned into the HindIII and XbaI sites of pHSS6 (28), forming pKC16. pKC1 was digested with NheI and SmaI, and then the

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TABLE 1. Strains and plasmids used in the study

Strain or plasmid	Properties	Reference
Plasmids		
pHSS6	*E. coli* cloning vector (Kan^r)	28
pIDN1	*N. gonorrhoeae* insertion/duplication plasmid (Erm^r)	15
pIDN3	*N. gonorrhoeae* insertion/duplication plasmid (Erm^r)	15
pKC1	*N. gonorrhoeae* insertion/duplication, positive/negative selection plasmid (Erm^r/Str^s)	3
pKC3	*ltgA* in pHSS6 (Kan^r)	3
pKH61	*ltgA* deletion plasmid (Kan^r)	This work
pKH82	*ltgA* deletion in pIDN1 (Erm^r)	This work
pKH84	pKH82 plus flanking sequence (Erm^r)	This work
pKH75	*ltgE* insertion/duplication plasmid (Erm^r)	This work
pKC4	*ltgA* disruption plasmid (Erm^r/Str^s/Kan^r)	3
pKC12	*ltgD* PCR product digested with EcoRV/BclI cloned into BamHI, EcoRV sites of pKC1 (Erm^r/Str^s)	This work
pKC14	*ltgD* internal deletion plasmid, formed by SmaI digestion of pKC12 (Erm^r/Str^s)	This work
pKC16	*ltgD* internal deletion plasmid, *ltgD* region subcloned into HindIII/XbaI sites of pHSS6 (Kan^r)	This work
pKC18	*ltgD* disruption plasmid, *ermC*/*rpsL* cloned into BglI site of pKC16 (Kan^r/Erm^r/Str^s)	This work
N. gonorrhoeae strains		
MS11	Wild type (Str^r)	31
KC119	MS11 *ltgD*	This work
KH560	MS11 *ltgA ltgD*	This work
KH571	MS11 *ltgE*	This work
ND500	MS11 with the GGI deleted	14

ermC/*rpsL* region was blunted with T4 DNA polymerase and ligated into the BglI site (blunted) of pKC16, forming pKC18. An insertion/duplication construct for mutation of *ltgE* was constructed by PCR amplification. Primers 5′-CTGAAGC TTGCAGCAACCATGCGTTTGAC-3′ and 5′-GCACTAGTAACGGGAGGC AGATACAACA-3′ were used to amplify a fragment of *ltgE*. The *ltgE* PCR product was restriction endonuclease digested with BspEI and FspI and then ligated into the Ecl136II and XmaI sites of pIDN3 (15), forming plasmid pKH75. Plasmid construction was verified by restriction endonuclease mapping.

Construction of gonococcal mutants. A construct containing *ltgD* disrupted with the *ermC*/*rpsL* cassette in pKC18 was used to transform MS11. To replace the positive-negative selection cassette with an internal deletion of *ltgD*, integrants were transformed with pKC16 linearized by EcoRI. Transformants were selected for streptomycin resistance and screened for erythromycin sensitivity. Mutation of *ltgD* in the MS11 background was confirmed by PCR, and the strain was named KC119. To construct MS11 *ltgA ltgD*, an in-frame start-to-stop deletion of *ltgA* was created in plasmid pKC3 (3) by amplification with primers 5′-TGAACGGGTCTCAGTCACATCGGATTTCCTTAAGAATCGGAAC-3′ and 5′-TCTAGCGGTCTCATGACGTGCCGATGCCGTCTG-3′ (restriction sites are underlined) followed by digestion with BsaI and ligation to form pKH61. The deletion construct region was excised from pKH61 with NotI (blunted) and EcoRI and was ligated into pIDN1 (15) digested with EcoRI and EcoRV to generate pKH82. To increase the homologous recombination frequency of the *ltgA* deletion construct with the gonococcal chromosome, a 181-bp region 3′ to *ltgA* was amplified from MS11 chromosomal DNA with primers 5′-GCGAATTCAACCATAAATATAAGACAATC-3′ and 5′-GACTGCGGC CGCCCATCATATCGGTGGAAAGGGTA-3′ and ligated into pKH82 at the EcoRI and NotI sites to generate pKH84. *ltgD* deletion strain KC119 was transformed with pKC4 to insert an *ermC-rpsL* marker into *ltgA* as previously described (3). Erythromycin-resistant transformants were selected and screened for streptomycin sensitivity. One such transformant was subsequently transformed with NsiI-digested pKH84, and Str^r transformants were selected. One Str^r Erm^s transformant, KH560, was selected for further study. Southern blotting demonstrated that KH560 was deleted for *ltgA* and *ltgD* as expected (see Fig. S1 in the supplemental material). An *ltgE* mutation was introduced into MS11 by transformation with pKH75 and selection for Erm^r colonies. The interruption in *ltgE* was confirmed by PCR.

Characterization of released peptidoglycan fragments. Gonococcal PG was purified and characterized following the methods of Rosenthal and Dziarski (25) as described by Cloud and Dillard (3). Briefly, log-phase gonococci were suspended at an OD_{540} of 0.2 in GCBL medium lacking glucose and containing 0.4% pyruvate, 0.1% glutamine, 0.0002% thiamine pyrophosphate, 0.0005% ferric nitrate, and 0.042% $NaHCO_3$. [6-^{3}H]glucosamine was added at a concentration of 2 μCi/ml, and the cells were grown for 2 hours. Cells were washed in GCBL medium and grown for 2.5 h in GCBL medium containing glucose and without label. Supernatants were harvested by centrifugation and applied to 350-ml Bio-Gel P6 and Bio-Gel P30 size exclusion columns connected in tandem. The columns were eluted with 0.1 M LiCl and 3-ml fractions collected. ^{3}H content was determined by liquid scintillation counting with 300 μl of each fraction.

Characterization of cytoplasmic peptidoglycan fragments. To examine PG fragments in the cytoplasm generated through PG recycling, the cell wall was pulse-labeled with [6-^{3}H]glucosamine as described above, and hot water extracts were produced as described by Garcia and Dillard (13). PG fragments in the extracts were separated by size exclusion chromatography and detected by scintillation counting.

RESULTS

Multiple lytic transglycosylases are present in *N. gonorrhoeae*. *N. gonorrhoeae* encodes seven lytic transglycosylase homologues (Table 2). To determine which of these enzymes act

TABLE 2. Seven lytic transglycosylases are encoded in the gonococcal genome

Gonococcal lytic transglycosylase	Function	*E. coli* homologue	% Identity/range (amino acids)	Meningococcal homologue
LtgA (NGO2135)	PG monomer production	Slt70	25/556	+[a]
LtgB (NGO1033)	Unknown	MltC	33/199	+
LtgC (NGO2048)	Cell separation	MltA	21/441	GNA33
LtgD (NGO0626)	PG monomer production	MltB	48/123	MltB
LtgE (NGO0608)	Unknown	MltD	34/341	+
LtgX[b] (NGO5004)	Type IV secretion	F-plasmid ORF169	33/129	+/−[c]
AtlA[b] (NGO5025)	Type IV secretion	Lambda endolysin	38/158	−[d]

[a] Homologue present.
[b] GGI-encoded lytic transglycosylase.
[c] Homologue present in some strains.
[d] Homologue not detected.

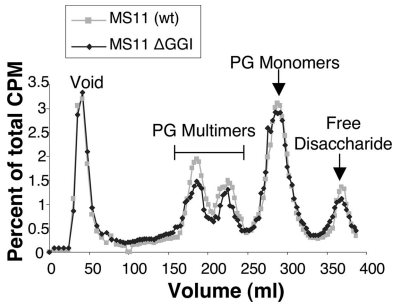

FIG. 1. Profile of PG fragments released by GGI deletion strain ND500 and wild-type parent strain MS11. ^{3}H-labeled PG fragments released into the culture medium were separated by size exclusion chromatography and detected by scintillation counting.

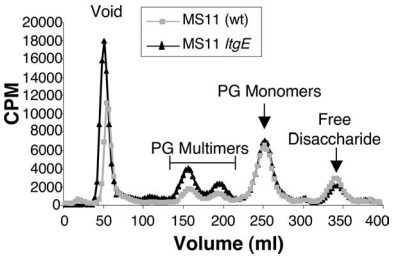

FIG. 2. Profile of PG fragments released by *ltgE* insertion mutant KH571 or wild-type parent strain MS11. Released ^{3}H-labeled PG fragments were separated by size exclusion chromatography and detected by scintillation counting.

in the production of cytotoxic PG fragments, we systematically mutated the gonococcal genes for the lytic transglycosylases and tested the mutants for PG fragment release. Previous work demonstrated that LtgA, the homologue of *E. coli* Slt70, was responsible for approximately half of the PG monomers released (3). Mutation of *ltgB* or *ltgC* did not affect monomer release (4, 19). A search of the gonococcal genome sequence of strain FA1090 identified two additional putative lytic transglycosylases, which we named LtgD and LtgE. The gonococcal genetic island (GGI), present in 80% of gonococcal strains but not in sequenced strain FA1090 (9), contains two more lytic transglycosylase homologues, AtlA and LtgX (14). We recently demonstrated that mutation of *atlA* does not affect PG fragment release (20).

Factors encoded in the gonococcal genetic island are not required for PG fragment release. The gonococcal genetic island carries 61 open reading frames and encodes a type IV secretion system and two lytic transglycosylase homologues as well as other proteins (14). Viala and coworkers identified a link between type IV secretion function and PG monomer release in *H. pylori* (34), leading to the possible conclusion that PG fragments might be transported by the type IV secretion system (7). However, comparison of wild-type gonococcal strain MS11 and its isogenic GGI deletion mutant (ND500) showed that the GGI deletion mutant was not deficient in PG monomer release (Fig. 1). The two strains were pulse-labeled by growth in medium containing [6-^{3}H]glucosamine to label the PG. PG fragments released into the supernatant were separated by size exclusion chromatography. Radioactivity in the samples was detected by scintillation counting and was normalized to total radioactivity released to correct for differences in the amount of label incorporated in the different cultures. No differences were seen in the profiles of released PG fragments (Fig. 1). This result indicates that neither the type IV secretion system components nor the GGI-encoded lytic transglycosylases AtlA and LtgX are necessary for PG fragment release in culture.

Mutation of *ltgE* does not decrease PG monomer release. A putative lytic transglycosylase gene (*ltgE*) encoding a protein similar to *E. coli* MltD was identified in the gonococcal chromosome by sequence comparisons (Table 2). An analysis of *H. pylori* mutants deficient in the LtgE homologue (MltD) indicated that the *H. pylori* enzyme acts as an endo-type lytic transglycosylase, shortening chain length and increasing anhydro ends in intact cell walls (2). Such an activity might generate more substrates for exo-type lytic transglycosylases and increase anhydro PG monomer production. An *ltgE* insertion mutation was constructed in gonococcal strain MS11 by transformation with a nonreplicating plasmid containing an internal fragment of *ltgE*. The PG of the wild-type and *ltgE* strains was pulse-labeled as before. For a direct quantitative comparison of PG fragments released, radioactivity in the cell pellet was determined after the labeling step and the cells were diluted to an equivalent amount of radioactivity for each culture for the chase period. Released PG fragments were analyzed by size exclusion chromatography (Fig. 2). Mutation of *ltgE* did not alter PG monomer release. However, PG multimer release appeared slightly increased in the mutant. These results suggest that LtgE is not significantly involved in PG monomer production.

Lytic transglycosylase D acts in PG monomer production. *N. gonorrhoeae* also encodes a putative lytic transglycosylase similar to *E. coli* membrane-bound lytic transglycosylase B (MltB) (Table 2). We designated this protein lytic transglycosylase D (LtgD). LtgD is predicted to be a lipoprotein of 363 amino acids. The catalytic residues (Glu162, Ser216, and Asn339), PG binding residues (Arg187, Phe226, Tyr259, and Tyr338), and amino acids lining the hydrophobic pocket (Ile158, Gln225, Tyr338, and Tyr344) of *E. coli* MltB, determined in biochemical and crystallographic studies (11, 33), are conserved in the LtgD sequence (data not shown).

In order to determine if LtgD acts in the release of PG monomers, an *ltgD* deletion mutation was made. The positive/negative selection method of Johnston and Cannon was used to generate an internal deletion in *ltgD* (17). In the first transformation, *ltgD* was interrupted with an *ermC*/*rpsL* cassette. In a subsequent transformation, an allele of *ltgD* containing a 1,000-bp deletion was introduced, generating an MS11 *ltgD* mutant (KC119) containing no antibiotic resistance markers.

Analysis of PG fragments released by *ltgD* deletion mutant KC119 demonstrated that *ltgD* is required for a substantial portion of PG monomer production. KC119 was metabolically labeled with [6-^{3}H]glucosamine, and released PG fragments were characterized by size exclusion chromatography. The resulting profile differed significantly from that of wild-type strain MS11. The *ltgD* mutant released less PG monomer and more PG multimers than wild type (Fig. 3). These data demonstrate that LtgD acts in the production of PG monomers and

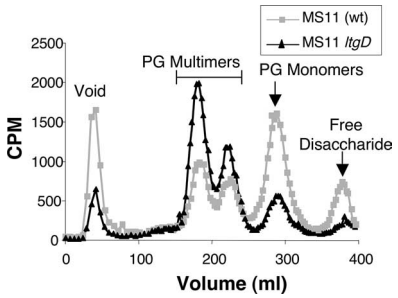

FIG. 3. Profile of PG fragments released by *ltgD* deletion mutant KC119 or wild-type parent strain MS11. ^{3}H-labeled PG fragments released into the culture medium were separated by size exclusion chromatography and detected by scintillation counting.

suggest that LtgD may function to degrade larger PG fragments liberated from the cell wall by other enzymes.

An *ltgA ltgD* double mutant does not release PG monomers. The previous results with an *ltgA* mutant (3) and the results shown here for the *ltgD* mutant (Fig. 3) indicate that each of these two putative lytic transglycosylases contributes substantially to PG monomer production. To determine if these are the only lytic transglycosylases required for PG monomer release, we introduced an *ltgA* mutation into the *ltgD* mutant. An in-frame deletion of the *ltgA* coding sequence was created, and this construct was introduced into *ltgD* mutant KC119. The presence of the two deletions in the resulting mutant KH560 was confirmed by Southern blotting (see Fig. S1 in the supplemental material). Although mutation of some gonococcal peptidoglycanases alters cell separation and growth characteristics (4, 12), mutation of *ltgA* and *ltgD* had no significant effects on in vitro growth of *N. gonorrhoeae* in either complex or defined medium (data not shown).

PG monomer release was evaluated in the *ltgA ltgD* double mutant with the pulse-chase labeling method and characterization of the released fragments by size exclusion chromatography. The profile of the released fragments showed no peak for PG monomers, suggesting that the *ltgA ltgD* mutant does not produce these cytotoxic PG fragments (Fig. 4). The profile for the mutant also showed an increase in release of larger, soluble PG fragments. The increased release of PG multimers is similar to that of the single *ltgA* or *ltgD* mutants, but more pronounced. This result suggests that the *ltgA ltgD* mutant is

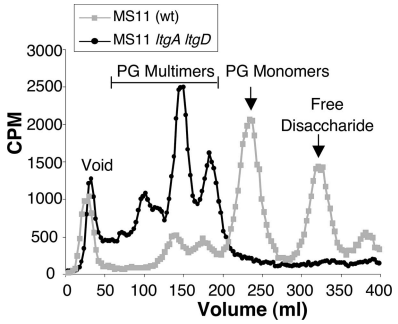

FIG. 4. Loss of PG monomer release by the MS11 *ltgA ltgD* double mutant. ^{3}H-labeled PG fragments released into the culture medium were separated by size exclusion chromatography.

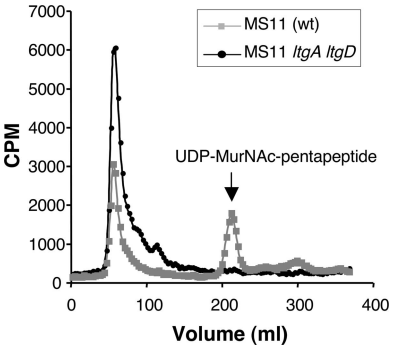

FIG. 5. Detection of recycled PG fragments in the cytoplasm of *ltgA ltgD* mutant KH560 or wild-type parent strain MS11. Size exclusion chromatography of hot water extracts shows the presence of the biosynthesis intermediate UDP-MurNAc-pentapeptide in the wild-type strain but its absence from the *ltgA ltgD* mutant.

able to degrade macromolecular PG to soluble fragments during growth but unable to break them down into monomers.

In addition to not releasing PG monomers, the *ltgA ltgD* mutant also showed no release of free disaccharide. Free disaccharide production requires the action of the amidase AmiC and is predicted to also require the function of a lytic transglycosylase (4, 12). The loss of free disaccharide release by the *ltgA ltgD* mutant as well as the reduced release of free disaccharide by the individual *ltgA* and *ltgD* mutants suggest that these two lytic transglycosylases may act in the generation of free disaccharide.

An alternative explanation for the absence of free disaccharide release in the *ltgA ltgD* mutant is that the mutant is not able to recycle the larger PG fragments and has increased metabolism of free disaccharide, as was demonstrated for a gonococcal *ampD* mutant (13). To test this hypothesis we repeated the pulse-chase experiment and looked for PG fragments in the cytoplasm (Fig. 5). The wild-type strain showed a single peak in the included volume corresponding to UDP-MurNAc-pentapeptide. The presence of labeled material in this PG biosynthesis precursor indicates that PG fragments were liberated from the cell wall and brought back into the cytoplasm for reuse, by the normal PG turnover and recycling processes. The absence of this peak in the *ltgA ltgD* mutant suggests that the mutant does not recycle liberated PG fragments.

DISCUSSION

The data presented here show LtgA and LtgD are responsible for PG monomer release by *N. gonorrhoeae*. These are two putative lipoproteins predicted to be inserted into the inner leaflet of the outer membrane (32). Gonococci have five other lytic transglycosylases that are not involved in PG monomer production (Table 2). It has been suggested that the lytic transglycosylases of *E. coli* may have overlapping functions and that one may substitute for the loss of another (21). That is not the case in *N. gonorrhoeae*. Gonococcal lytic transglycosylase C, LtgC, is the only lytic transglycosylase required for normal growth characteristics, being required for cell separation after division (4). The lytic transglycosylases encoded in the GGI are required for type IV secretion of DNA but do not affect PG

monomer production or cell growth (20). The functions of LtgB and LtgE remain a mystery (19), although mutation of *ltgE* has a slight effect on PG dimer release (Fig. 2), a result consistent with its possible role as an endolytic transglycosylase as predicted from *H. pylori* studies (2).

LtgA and LtgD homologues may also be required for PG monomer release in other bacterial species and thus affect infection phenotypes. Mutation of the *ltgA* homologue has been shown to reduce PG monomer production in several other bacterial species. An *H. pylori ltgA* homologue mutant is reduced 40% in monomer release and induces less interleukin-8 (IL-8) production in human (HEK293) cells (34). Likewise, *Shigella flexneri* requires an *ltgA* homologue for full virulence, with the mutant showing reduced inflammation in the guinea pig conjunctivitis model (1). LtgD homologues are found in species known to release PG monomers or where PG monomers have been demonstrated to affect infection phenotypes, including *S. flexneri* (GenBank accession no. ABF 04888.1), *B. pertussis* (NP 879760.1), and *Vibrio fischeri* (AAW86197.1).

It is not too surprising that the GGI-encoded lytic transglycosylases AtlA and LtgX are not required for PG monomer release into the medium, since these enzymes were shown to function in type IV secretion of DNA (20). Secretion system lytic transglycosylases are thought to create a small opening in the cell wall in order to allow for assembly of the secretion apparatus and would thus be expected to have a localized and limited function (8). However, it was shown that *H. pylori* mutants defective in the type IV secretion system induced less IL-8 production and less Nod1 signaling (34). It was suggested that PG fragments might pass through or be transported through the type IV secretion system (7). It is not known how PG fragments traverse the outer membrane in *N. gonorrhoeae*, but it is clear from our results that the type IV secretion system is not necessary for the transport. As PG monomers are small (921 Da for the anhydro disaccharide tetrapeptide monomer), they may simply diffuse across the outer membrane. This scenario does not exclude the possibility that the type IV secretion system could enhance transport of PG fragments into a host cell during infection.

It is not clear why gonococci would need two different enzymes, LtgA and LtgD, to perform what is apparently the same function. One possibility is that the functions they perform are not exactly the same. LtgA and LtgD may have different substrate specificities or act in coordination with other enzymes. For example, maybe LtgA degrades PG strands that are not cross-linked, while LtgD might act on cross-linked PG and require an endopeptidase for monomer liberation. Maybe the two enzymes perform the same reaction but at different places in the cell. One might degrade PG at the poles and the other in the expanding side wall. Further characterizations will be necessary to identify differences in LtgA and LtgD functions.

Why do gonococci have these lytic transglycosylases at all? The *ltgA ltgD* double mutant showed no abnormalities in growth or cell separation. Thus, it appears that gonococci do not need these enzymes. What then is their purpose in the cell, and why do gonococci have not just one, but two genes for this function? One possibility is that they contribute to infection processes. Clearly, released PG fragments modulate the host immune response and kill ciliated cells of the fallopian tubes (10, 23). Killing of ciliated cells could help provide a niche for gonococcal growth and proliferation, eliminating cilial beating and allowing access for the bacteria to subepithelial tissue. Also, it has been suggested that sterility in women, such as tubal factor infertility caused by *N. gonorrhoeae*, at least in ancient times, may have led to increased numbers of sexual encounters and thus increased the spread of sexually transmitted diseases (22). Spread of *N. gonorrhoeae* may also be enhanced by a strong inflammatory response in men and women. Induction of IL-8 by PG fragments would lead to a large influx of neutrophils into the site of infection. Gonococci can survive in neutrophils (29) and may be transmitted to the next individual in these cells in the purulent discharge typical of gonococcal infection.

The absence of free disaccharide release by the *ltgA ltgD* double mutant suggests another reason for the presence of these enzymes, i.e., for PG recycling. Gonococci have an efficient PG recycling system that takes up and reuses 85% of the PG fragments generated during growth in vitro (13). The PG fragments taken up into the cytoplasm are the monomers and, to a lesser extent, free disaccharide, i.e., those generated by the action of lytic transglycosylases. A mutant deficient in PG monomer recycling, as demonstrated by the gonococcal *ampD* mutant, shows virtually no release of free disaccharides even though it produces the same amount as the wild type. The *ampD* mutant was shown to have increased uptake and metabolism of free disaccharide in response to the recycling deficiency, using up nearly all the free disaccharide instead of releasing it (13). Since the *ltgA ltgD* mutant does not produce PG monomers (Fig. 4), then the decreased PG fragment recycling may similarly result in increased metabolism of free disaccharide. The function of lytic transglycosylases may be to provide PG fragments that can be recycled. The lack of recycled PG fragments in the *ltgA ltgD* cytosol indicates that this mutant is deficient in PG recycling (Fig. 5). PG recycling would allow for recovery of nutrients, the ability to sense the growth state of the cell wall, and the possible ability to regulate the release of PG fragments.

In summary, systematic mutagenesis has demonstrated that *N. gonorrhoeae* uses two of its seven lytic transglycosylases for production of cytotoxic PG monomers. These enzymes are not necessary for normal growth but do affect the ability of the bacterium to recover liberated PG fragments for PG recycling.

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