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Mutation of a Single Lytic Transglycosylase Causes Aberrant Septation and Inhibits Cell Separation of *Neisseria gonorrhoeae*

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The function of lytic peptidoglycan transglycosylases is poorly understood. Single lytic transglycosylase mutants of *Escherichia coli* have no growth phenotype. By contrast, mutation of *Neisseria gonorrhoeae ltgC* inhibited cell separation without affecting peptidoglycan monomer production. Thus, LtgC has a dedicated function in gonococcal cell division.

Lytic transglycosylases act to cleave peptidoglycan (PG) and are thought to function in the removal of PG strands for cell wall remodeling during bacterial growth and division (9). However, mutations in single or multiple lytic transglycosylase genes do not inhibit growth or division in *Escherichia coli* (11). Only a strain with all known lytic transglycosylases mutated was reported to have a slight division phenotype, growing in groups of approximately three to eight cells (8). *Neisseria gonorrhoeae* is a gram-negative coccus that normally grows as a diplococcus or single coccus and releases soluble PG fragments during growth. The major fragments released are the 1,6-anhydrodisaccharide tripeptide monomer and the 1,6-anhydrodisaccharide tetrapeptide monomer (16). These PG fragments have potent biological effects, including killing ciliated fallopian tube cells (13), inducing inflammatory cytokine production (4), and causing arthritis (5). Previous studies in our laboratory found that mutations affecting lytic transglycosylases LtgA and LtgB lower PG monomer production but do not alter cell division (2; K. A. Cloud, E. T. Beck, and J. P. Dillard, submitted for publication). We are mutating and characterizing putative lytic transglycosylases in order to determine which enzymes are responsible for PG fragment production and release.

Identification of *N. gonorrhoeae* lytic transglycosylase C. *N. gonorrhoeae* encodes a membrane-bound lytic transglycosylase A (MltA) homologue, which we have designated lytic transglycosylase C (LtgC). LtgC exhibits 21% identity and 31% similarity to *E. coli* MltA and contains a consensus lipoprotein site, suggesting that, like many other PG hydrolases, LtgC may be a lipoprotein. A close homologue of LtgC (known as GNA33) has been studied in *Neisseria meningitidis*. Jennings et al. determined that GNA33 has lytic transglycosylase activity and is a lipoprotein when expressed in *E. coli* (10).

Creation of an *ltgC* mutant and complemented strain. A combination of positive and negative selection was used to create a 33-bp deletion at the 5′ end of *ltgC* that removed the putative start codon. *ltgC* was amplified from *N. gonorrhoeae* MS11 chromosomal DNA by using specific primers 5′ ATT GCCTGCCGCCGGTTTATAG 3′ and 5′ AAGAAACGCC ATACCGACCAAG 3′ and inserted into pKC1 (2), forming pKC11 (Table 1). Through several steps, an *ermC-rpsL* cassette was inserted into an internally deleted *ltgC*, forming pKC17. This plasmid was transformed into *N. gonorrhoeae* by the method of Gunn and Stein (6), and transformants were selected with 10 μg of erythromycin/ml. A deletion in the 5′ end of *ltgC* was formed by digesting pKC11 with BsaXI, blunting it with T4 DNA polymerase, and ligating the DNA to form pKC19. This plasmid was transformed into the *ltgC* insertion strain in order to replace the *ermC-rpsL* cassette with the *ltgC* deletion. Streptomycin resistance at 100 μg/ml was used to select for loss of the original insertion.

A complemented strain was constructed by inserting a wild-type copy of *ltgC* at a distant location on the gonococcal chromosome. To create the complementation construct, *ltgC* and 211 bp of 5′ DNA were amplified by PCR with primers 5′ GACTAGTGACGGGCTTCGGACGGCA 3′ and 5′ GCGA TGCATTAAACGCGAATGAACAAGG 3′ and cloned into pKH23, forming pKC22. The complementation plasmid is derived from pGCC6 and allows incorporation of the introduced gene into the gonococcal chromosome between *aspC* and *lctP* (12). Following transformation of KC118 with pKC22 and selection with chloramphenicol at 10 μg/ml, the desired strain (KC124) was identified using PCR by screening for both retention of the *ltgC* mutation and incorporation of *ltgC* at the alternate location.

Mutation of *ltgC* affects gonococcal growth. Colonies of KC118 appeared slightly smaller on an agar plate, and the mutant did not appear to grow rapidly when inoculated into liquid culture. Gonococci were grown with aeration in gonococcal base liquid medium as previously described (2). When inoculated at equivalent optical densities, both the number of CFU per milliliter (Fig. 1B) and the optical density of the mutant culture (Fig. 1A) were below those of the wild-type and complemented strains. Measurement of total protein in the cultures by the Bio-Rad protein assay showed that MS11*ltgC* and wild-type strains accumulated equivalent amounts of protein during the initial 4 h of log phase. Thus, the protein accumulation in the culture reflected the fact that the cells were growing to equivalent levels, whereas the numbers of CFU per milliliter differed by 5- to 10-fold. These results suggested that mutation of *ltgC* resulted in decreased cell viability or prevented cell division.

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Properties	Reference or source
pHSS6	*E. coli* cloning vector (Kan^r)	15
pKH23	Gonococcal complementation vector (Cam^r) derived from pGCC6	K. T. Hackett
pKC1	*N. gonorrhoeae* insertion-duplication, positive-negative selection plasmid (Erm^r Str^s)	2
pKC11	*ltgC* PCR fragment (MfeI; NruI) ligated into pKC1 at EcoRI and SmaI sites (Erm^r Str^s)	This work
pKC13	*ltgC* internal deletion; pKC11 digested with StyI (Erm^r Str^s)	This work
pKC15	*ltgC* internal deletion subcloned into pHSS6 (Kan^r)	This work
pKC17	*ltgC* disruption plasmid; *ermC-rpsL* cloned into FseI site of pKC15; Kan^r Erm^r Str^s	This work
pKC19	5′ deletion of *ltgC*; pKC11 digested with BsaXI, blunted (Erm^r Str^s)	This work
pKC22	*ltgC* complementation vector; ltgC cloned into NheI and NsiI sites of pKH23 (Cam^r)	This work
MS11	Wild-type *N. gonorrhoeae* (Str^r)	14
KC118	MS11*ltgC*	This work
KC124	MS11*ltgC* complemented (Cam^r)	This work

The differences in viable gonococci seen at various time points between MS11 and MS11*ltgC* could be due to an increased level of cell lysis in the mutant. MS11*ltgC* was found to undergo autolysis more than the wild type. Bacterial viability was determined by using a Live/Dead BacLight bacterial viability kit (Molecular Probes). Cultures were diluted to an optical density at 540 nm of 0.3, equivalent to 1.2×10^8 CFU/ml for the wild-type strain. Aliquots were washed in 0.1 M MOPS (morpholinepropanesulfonic acid)–1 mM $MgCl_2$ (pH 7.2). After 2 h of growth, threefold-more dead bacteria were present in cultures of MS11*ltgC* (24.1% dead) than in those of the wild type (7.9% dead). Complementation of *ltgC* restored the wild-type phenotype; similar numbers of dead bacteria were seen in cultures of KC124 (4.2% dead) and MS11 (data not shown).

Mutation of *ltgC* alters septation and cell separation in *N. gonorrhoeae*. A disruption in cell division or separation would be one explanation for the decreased level of CFU per milliliter seen in cultures of MS11*ltgC*. To investigate this possibility, gonococci were grown overnight on gonococcal base plates and thin sections of each strain were prepared for transmission electron microscopy as described by Mehr et al. (12). Mutation of *ltgC* altered septation and division in *N. gonorrhoeae*, resulting in septa that were wavy (Fig. 2A) and thickened (Fig. 2B). Cells of MS11*ltgC* did not separate properly (Fig. 2B and E). Similar to the cell viability staining results, more lysed cells were seen in the preparations of MS11*ltgC* than in those of the wild type (Fig. 2E). Complementation of *ltgC* reversed these phenotypes (Fig. 2C and F); the morphology of the complemented strain was indistinguishable from that of MS11 (Fig. 2D). To quantify the cell separation defect, 500 cells of MS11, MS11*ltgC*, and the complemented strain were viewed. The number of cells per group and the number of groups of that size were counted (Fig. 3). Cells of MS11 and the complemented strain were mainly found growing in groups of one or two cells. Cells of MS11*ltgC* were mainly present in groups of

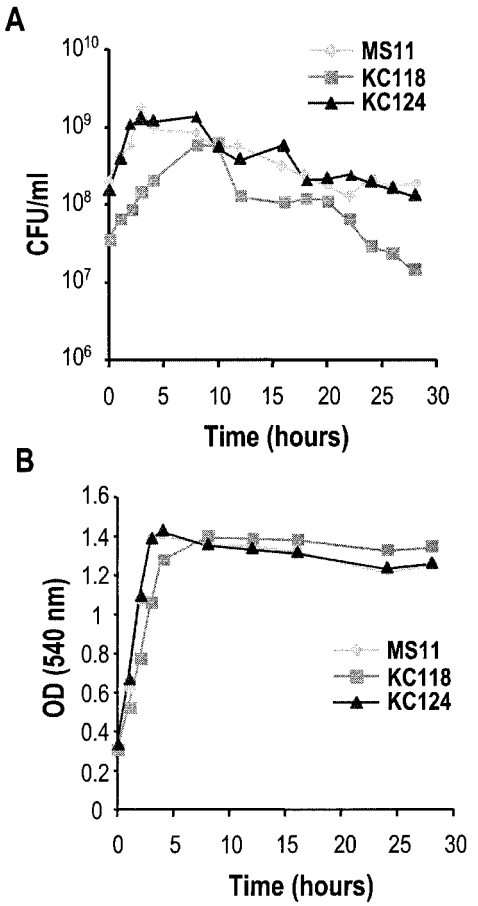

FIG. 1. Mutation of *ltgC* affects growth characteristics. Aliquots of MS11, MS11*ltgC*, and MS11*ltgC* (complemented) cultures were taken, and CFU per milliliter (A) and optical density at 540 nm (B) were determined. Graphs shown are representative of three separate trials.

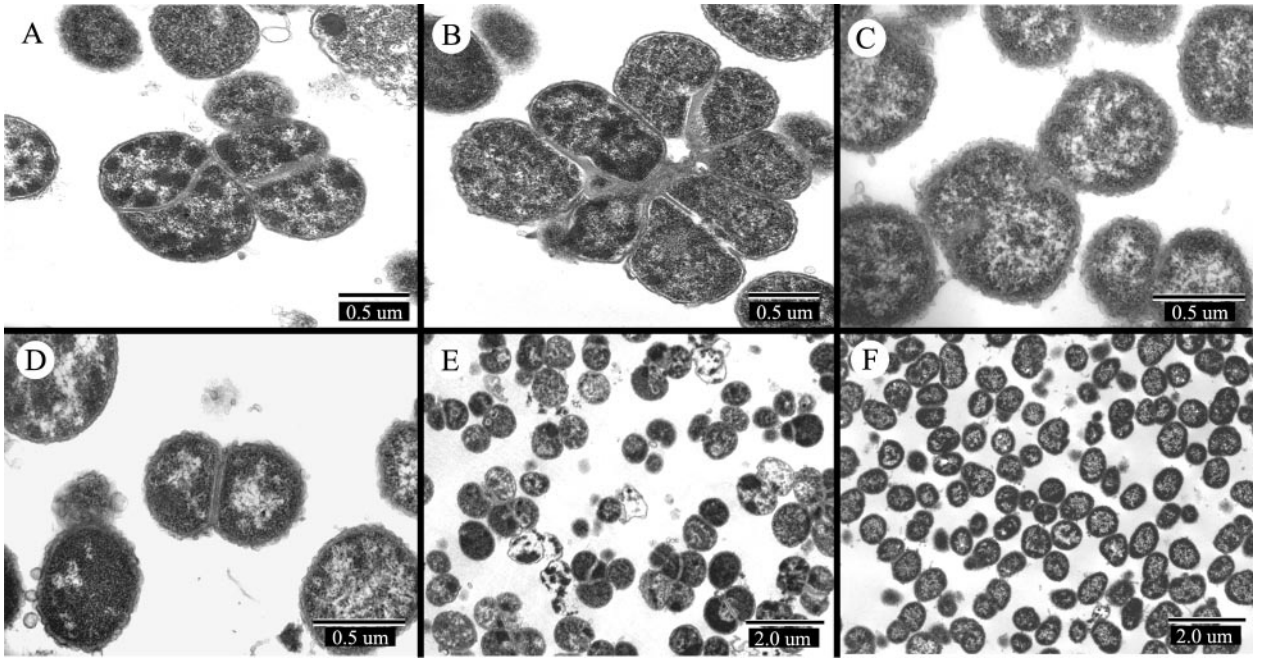

FIG. 2. Mutation of *ltgC* alters septation and cell separation. Thin-section transmission electron micrographs of MS11*ltgC* show irregular, wavy septa (A) or incomplete cell separation and thickened septa (B). Micrographs of MS11*ltgC* (complemented) (C) show that it is indistinguishable from the wild-type cell morphology of MS11 (D). Low-magnification micrographs of MS11*ltgC* (E) demonstrate that a majority of cells show cell separation defects; these are not seen in the *ltgC*-complemented strain (F).

three to five cells, and this was the only strain for which groups containing more than 10 cells were seen. Since thin sections were used for this analysis, additional bacteria outside the plane of the section were missed. Therefore, the number of bacteria in the aggregates is likely to exceed those counted.

Effects of *ltgC* mutation on PG fragment release. To determine if LtgC functions in the production or release of PG monomers, PG from MS11 and KC118 was metabolically labeled with [6-^{3}H]glucosamine, and released PG fragments were collected and analyzed by size-exclusion chromatography as previously described (2). PG monomer release was not significantly reduced in the *ltgC* mutant, showing at most a slight decrease (Fig. 4A). Similarly, larger PG fragments were slightly increased. However, the most striking difference from the wild-type profile was the absence of released free disaccharide in the MS11*ltgC* profile (Fig. 4A). Free disaccharide release was restored by complementation of *ltgC* (Fig. 4B). Free disaccharides are predicted to be released by the combined action of a lytic transglycosylase and an amidase (7). Therefore, this result suggests that either the *ltgC* mutant is deficient in amidase activity or LtgC must first cleave the glycosidic bond before an amidase can act to remove the disaccharide from the peptide.

If LtgC acts as a major contributor to PG fragment release, then *ltgC* mutants should show a lower rate of PG turnover, and more of the original PG should remain in the macromolecular PG than is maintained by the wild-type strain. However, soluble PG fragments were released into the medium at a higher rate in the *ltgC* mutant than in the wild type. PG turnover was measured for the wild-type, *ltgC* mutant, and complemented strains as described previously (2). After 4 h, only 51.2% ± 4.3% of macromolecular PG remained in KC118. This value was significantly different from that of the wild type (66.1% ± 8.0%) or the complemented strain (76.6% ± 3.3%) as determined by Student's *t* test, $P < 0.05$. These values are the averages of three experiments, and the error values are the standard errors. Although it is possible that the rate of turnover is enhanced in the *ltgC* mutant, we suspect that cell lysis during the first 4 h of growth accounts for this result. The macromolecular PG in lysed cells may not be as efficiently recovered by centrifugation as in whole cells. The appearance of high-molecular-weight PG fragments between the void peak and the PG multimer peaks in the profile of the *ltgC* mutant (Fig. 4A) is consistent with this idea.

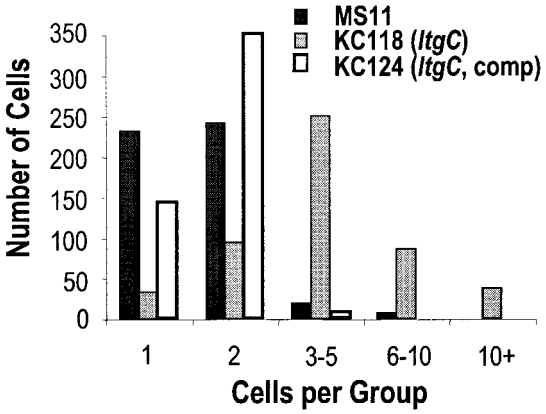

FIG. 3. Mutation of *ltgC* disrupts cell separation. Cells of MS11*ltgC* were seen growing in larger clusters than those of the wild type. Complementation of *ltgC* restored normal growth patterns.

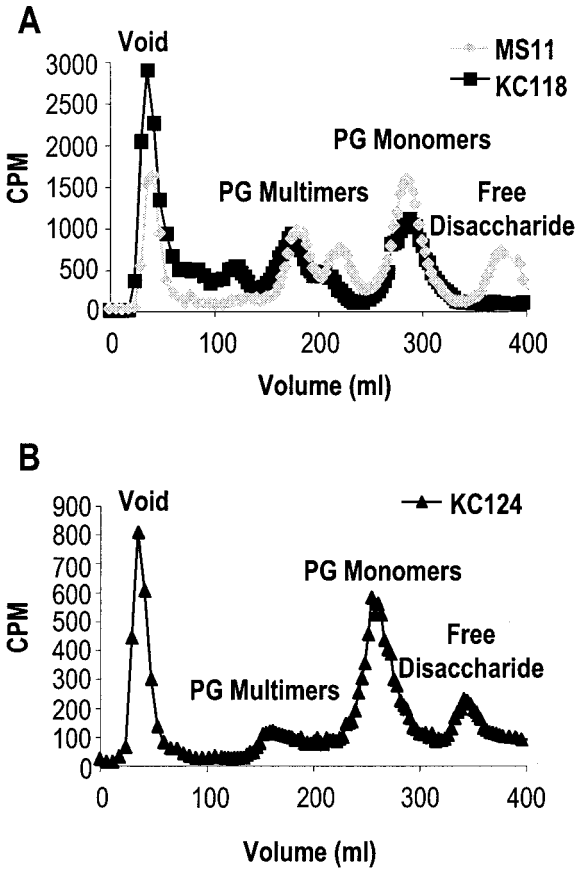

FIG. 4. Mutation of *ltgC* alters the profile of released PG fragments. (A) ^{3}H-labeled PG fragments were collected and separated by gel filtration chromatography. Mutation of *ltgC* inhibits the release of free disaccharide by growing gonococci. (B) Complementation of *ltgC* restores free disaccharide release.

Conclusions. Mutation of *ltgC* resulted in cells that did not separate, had abnormal septa, and exhibited abnormal growth characteristics. Normal septation, growth, and cell separation were restored by the addition of a wild-type *ltgC* at a distant location on the chromosome. Thus, it is clear that *ltgC* is required for normal cell division and separation processes. Given its similarity to known lytic PG transglycosylases, we predict that LtgC functions in the removal of PG strands for splitting of the cell wall during cell division. Because of the severity of the defect seen in the thin-section electron micrographs, it is somewhat surprising that the mutants are viable and capable of exponential growth. The high degree of autolysis seen in the *ltgC* mutant may allow cells to split off from a cluster, thereby facilitating cell separation. In support of this hypothesis, many of the MS11*ltgC* cells in Fig. 2E have attached, lysed cells.

Recently, a report was published describing the effects of mutation of *gna33* in *N. meningitidis* (1). The *gna33* mutants did not separate well, released outer membrane proteins into the culture to a higher degree than the wild type did, and were avirulent in a rat model of septicemia (1). These results in *N. meningitidis* are similar to ours, suggesting that GNA33 and LtgC likely perform similar functions in these closely related species. The high degree of autolysis seen in *ltgC* mutants may also occur in *gna33* mutants and could explain the additional protein release noted by Adu-Bobie et al (1).

The action of LtgC differs from that of the other gonococcal lytic transglycosylases that we have studied and from that of *E. coli* lytic transglycosylases. Mutation of *ltgC* does not greatly impact the release of PG monomers. Also, the other gonococcal lytic transglycosylase mutants are reduced, not increased, in autolysis (2, 3; Cloud et al., submitted). In *E. coli*, growth irregularities linked to the deletion of a single lytic transglycosylase have not been seen in the multiple investigations of these enzymes (11). By contrast, inactivation of LtgC alone alters gonococcal growth and inhibits cell separation. These data suggest that LtgC is a promising target for antimicrobials.

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