Characterization of the role of LtgB, a putative lytic transglycosylase in Neisseria gonorrhoeae

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Neisseria gonorrhoeae releases monomeric peptidoglycan (PG) fragments during growth. These PG fragments affect pathogenesis-related phenotypes including induction of inflammatory cytokines and killing of ciliated fallopian tube cells. Although the biological activities of these molecules have been established in multiple systems, the genes and gene products responsible for their production in N. gonorrhoeae have not been determined. The authors previously identified genes for three lytic transglycosylase homologues (ltgA, ltgB and ltgC) in the N. gonorrhoeae genome sequence. Mutation of ltgA was found to affect PG fragment release, and mutation of ltgC affected cell separation. In this study the effects of complete deletion or point mutations in ltgB were characterized. Point mutations were introduced by a combination of insertion-duplication mutagenesis and positive and negative selection, thereby generating selectable marker-less mutations. The ltgB deletion mutant had normal growth characteristics and was not affected in PG fragment release. When expressed in Escherichia coli, gonococcal LtgB was able to substitute for lambda endolysin to cause cell lysis. Mutation of the predicted catalytic-site glutamic acid residue did not decrease lysis in this system. However, mutation of a nearby glutamic acid residue eliminated lysis activity.

INTRODUCTION

Most bacteria encode multiple lytic peptidoglycan (PG) transglycosylases. Although the function of several of these enzymes produced by Escherichia coli or bacteriophages has been characterized biochemically (reviewed by Hölzle, 1998), the importance of this reaction to the bacterial cell and the need for multiple enzymes remain unclear. Lytic transglycosylases cleave the N-acetylmuramic acid–β-1,4-N-acetylglucosamine linkage in PG and catalyse the formation of a 1,6-anhydro bond on the N-acetylmuramic acid (Hölzle et al., 1975). Ostensibly, the biological function of these enzymes is to remove PG strands to allow for cell wall expansion during growth and for remodelling during cell division. However, E. coli mutants lacking several of the lytic transglycosylases are not affected in growth or division, and growth phenotypes are only seen in the presence of cell wall synthesis inhibitors or in strains containing mutations affecting PG-synthesizing enzymes (Lommatzsch et al., 1997). Recently, an E. coli mutant lacking all six lytic transglycosylases was shown to be slightly affected in cell separation and grew in short chains (Heidrich et al., 2002).

Liberated PG fragments contribute to virulence in infections caused by the bacterium Neisseria gonorrhoeae. Unlike most other Gram-negative bacteria, gonococci release soluble PG fragments into the surrounding milieu during growth. The major fragments released are the 1,6-anhydrosialic acid PG monomers, the type of fragments produced by lytic transglycosylases (Sinha & Rosenthal, 1980). These PG fragments have been shown to cause the sloughing of ciliated fallopian tube cells in the organ culture model of gonococcal pelvic inflammatory disease (PID) (Melly et al., 1984). PG fragments have also been shown to induce arthritis in a rat model, producing symptoms similar to the arthritis seen in patients with disseminated gonococcal infection (DGI) (Fleming et al., 1986). PG monomers have been shown to induce production of the inflammatory cytokines IL-1 and IL-6 in human monocytes (Dokter et al., 1994). Similar PG fragments are also involved in the pathogenesis of infections caused by Bordetella pertussis and Haemophilus influenzae. In B. pertussis, released monomeric PG fragments kill ciliated cells of the trachea (Luker et al., 1995). In a rabbit model of H. influenzae meningitis, various soluble PG fragments were shown to contribute to brain oedema and leukocytosis (Burroughs et al., 1993). These results suggest that PG fragments are involved in the pathogenesis of PID and DGI, and possibly the large inflammatory response characteristic of symptomatic, uncomplicated gonorrhoea.

Although the effects of PG fragments on biological systems have been well characterized, the genes involved in...
production and release of these fragments have not been identified in any pathogen known to produce them. It might be hypothesized that the lytic PG transglycosylases generate the fragments, but it is unclear why the fragments are not efficiently recycled as occurs in E. coli. (For a review of PG recycling, see Park, 1995.) Do gonococci simply produce more PG monomers than can be handled by the recycling apparatus, or does production occur in some manner that favours release rather than uptake? To better understand the production and release of PG fragments by N. gonorrhoeae, we have produced mutations in lytic transglycosylase genes. These mutations will allow us to assess the role and relative contribution of each gene product to PG monomer production and release of PG fragments by N. gonorrhoeae.

**METHODS**

**Bacterial strains and growth conditions.** Bacterial strains and plasmids used in this study are listed in Table 1. All experiments except transformations were performed with nonpiliated variants. Gonococci were grown with aeration in GC base liquid (GCBL) medium (1 % protease peptone no. 3, 0-4 % K2HPO4, 0-1 % KH2PO4, 0-1 % NaCl; pH 7-2) containing Kellogg’s supplements and 0-042 % NaHCO3, or on GCB agar plates (Difco) in 5 % CO2 at 37 °C (Kellogg et al., 1963; Morse & Bartenstein, 1974). E. coli was grown in Luria broth or on Luria agar plates (Sambrook & Dillard, 2002). Here we demonstrate that the putative lytic transglycosylase LtgB acts to degrade PG when produced in E. coli and can functionally replace the lytic transglycosylase of bacteriophage lambda. In addition, we describe mutations in LtgB and their effects on PG fragment release in N. gonorrhoeae.

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<th>Table 1. Strains and plasmids</th>
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<td><strong>Plasmid or strain</strong></td>
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<td>pKC1</td>
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<td>RLG4065</td>
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<td>MS11</td>
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(Tm 64°C, extension time 1-5 min). The PCR product was digested with NruI and HindIII and ligated to Small- and HindIII-digested pC1 to create pH58. The coding region of ltgB was removed by PCR amplification of the plasmid using primers lgtBmut1 (5’-CGGTAGCTAGTATTTGAAACGGGTGCCG-3’) and lgtBmut2 (5’-AGTCATGATTTTGAACCCGTGCCG-3’) (Tm 63°C, extension time 3 min). The resulting PCR product was digested with BspHI and religated to form pH60. The coding region was thus replaced by the sequence 5’-ATGTCATGA-3’, only encoding a methionine and a serine.

**ltgB mutants of N. gonorrhoeae.** N. gonorrhoeae strain MS11 (Segal et al., 1985) was transformed with pFK4 by the method of Gunn & Stein (1996) and transformants were selected on GCB-Erm plates. ErmR transformants were grown in GCBL with no antibiotic selection for 3 h and then plated on GCB-Str to select for the loss of the originally inserted plasmid. StrR colonies were transferred to GCB-Erm plates to verify loss of the plasmid from the chromosome. Presence of the mutation in ltgB was determined by PCR amplification of ltgB from mutant (PK106) chromosomal DNA and digestion with Asel. A deletion in ltgB was introduced into N. gonorrhoeae by transformation of strain MS11 with pH60. Transformants were screened for the ltgB deletion by PCR using primers lgtB1 (5’-CGGGTGCTGACGACGCGATTAT-3’) and lgtB2 (5’-CGCGAAACCATCACCACAAAG-3’). The resultant ltgB deletion strain was designated KH539.

**Construction of strains for lysis assay.** The bacteriophage lambda lysis genes S, R and Rz were PCR amplified from lambda lysogen RLG4065 using primers lysisF (5’-GGGATTCCTACAGTGACGTTAGACACACGACG-3’) and lambdalsyR (5’-GGGATTCCTAGCCGTAATGGAATTGCTGCGCCG-3’) \( \text{(Tm} 55-0^\circ\text{C, extension time 1 min)} \). The resulting PCR product and the vector, pBAD30 (Guzman et al., 1995), were digested with Xhol and EcoRI and ligated with T4 DNA ligase. Chemically competent E. coli were transformed with the ligation reaction. Transformants were grown overnight and AmpR colonies were screened for a plasmid of the expected size, 6-5 kb.

ltgB was PCR amplified directly from N. gonorrhoeae strain MS11 colonies with primers lgtBlisyF (5’-CGGTAGCTACATTGAAAAAACCGGACGTAACACC-3’) and lgtBlisyR (5’-CGGAGTCTAGCCGCCCAGTGACGATCCG-3’) \( \text{(Tm} 60-0^\circ\text{C, extension time 1 min)} \). In order to create a vector with the lambda genes S and Rz but not R, pPK20 was PCR amplified from the end of S upstream and from the beginning of Rz downstream using the primers SacIlysisF (5’-CGGTAGCTACATTGAAAAAACCGGACGTAACACC-3’) and lgtBlysisR (5’-CGGTAGCTACATTGAAAAAACCGGACGTAACACC-3’) (Tm 60-0°C, extension time 3 min). The resulting PCR products were digested with SacI and KpnI and ligated using T4 DNA ligase. The ligation reaction was transformed into E. coli as described above and AmpR colonies were screened for a plasmid of the expected size of pPK33. The plasmid was mapped by restriction digestion to confirm the insertion of ltgB. The plasmid pPK46, encoding S, Rz and LtgBE117A, was constructed using the same method as described for constructing pPK33 except that ltgB encoding the point mutation was PCR amplified from pPK44. The plasmid pPK39, encoding S, Rz and LtgBE117A, was also constructed using the same method as used to construct pPK33 except that ltgB encoding the point mutation was PCR amplified from pEB4.

**Lysis assay.** Strains were grown overnight in LB broth with Amp and then diluted to an OD\(_{550}\) of 0.6 in M9 minimal medium with glycerol and Amp. Cultures were induced with 0.2% L-arabinose and grown at 37°C with aeration. The OD\(_{550}\) was measured immediately upon induction and every 30 min thereafter for 300 min using a Beckman DU-64 spectrophotometer.

**Peptidoglycan assays.** PG purification methods and PG turnover assays were performed essentially as described by Cloud & Dillard (2002). For PG fragment release assays, PG was pulse-labelled with 2 μCi ml\(^{-1}\) (74 kBq ml\(^{-1}\)) [\(^{6}\text{H}\)]glucosamine (Amersham) in GCBL containing pyruvate as the carbon source. For each time point, the cultures were centrifuged, and the macromolecular PG was harvested using the boiling SDS method. Turnover was measured as loss of radioactivity from the macromolecular PG fraction over time. For characterization of released PG fragments, supernatants from exponential-phase cultures were applied to size-exclusion columns and fractions were assayed for radioactivity by scintillation counting. Statistical significance was determined by Student’s t test.

**RESULTS**

We previously described the identification of three lytic transglycosylation homologues in the gonococcal genome sequence. The first gene, designated ltgA, was characterized and found to be involved in PG monomer production (Cloud & Dillard, 2002). The second gene, designated ltgB, is characterized here. We recently showed that the third gene, ltcG, is involved in cell separation (Cloud & Dillard, 2004).

ltgB encodes a homologue of E. coli MltC. Although the effects of single mltC mutations have not been reported, purified E. coli MltC has been shown to have lytic transglycosylase activity in vitro (Dijkstra & Keck, 1996). LtgB is 31% identical and 47% similar to MltC over a 100 amino acid region, and the sequence motifs common to family 1 lytic transglycosylases are conserved in LtgB (Fig. 1) (Blackburn & Clarke, 2001). One difference between LtgB and MltC is that the LtgB predicted amino acid sequence does not contain a lipoprotein-processing site, and thus LtgB is not likely to be a membrane-bound lipoprotein as is MltC. In addition to the similarity to MltC, LtgB also shares over 90% amino acid identity with putative lytic transglycosylases in the two sequenced strains of Neisseria meningitidis. Examination of the N. gonorrhoeae genome sequence in the ltgB region shows that just upstream of ltgB is a copy of the insertion sequence IS1106, and immediately downstream of ltgB is a gene for a hypothetical transporter of the major facilitator family.

**LtgB can functionally replace the lytic transglycosylase in the bacteriophage lambda lysis system**

The homology of LtgB with MltC suggests that LtgB is a PG lytic transglycosylase. We investigated the ability of LtgB to function in PG degradation by using LtgB to replace the PG transglycosylase in the bacteriophage lambda lysis system. Lambda encodes three gene products necessary for lysis of the host cell during the lytic stage of the bacteriophage lifecycle, R, S and Rz (Young, 1992). R is the PG transglycosylase necessary for degradation of the cell wall during lysis (Bienkowska-Szewczyk et al., 1981). S is a holin, a small transmembrane protein necessary to allow entry of R into the periplasm (Young, 1992). The function of Rz is unclear, although the locus encodes two gene products, Rz and Rz1 (Hanych et al., 1993). We cloned S, R and Rz from lambda
and placed them under the control of an arabinose-inducible promoter to create the plasmid pPK20. Upon induction with arabinose, *E. coli* carrying pPK20 lysed rapidly (Fig. 2). We next constructed a plasmid containing *ltgB* instead of *R* in order to determine whether LtgB could functionally replace R in the lambda lysis system. The strain of *E. coli* carrying the plasmid containing *S*, *ltgB* and *Rz* (pPK33) lysed when arabinose was added to the culture medium, though not as quickly as the strain carrying the plasmid encoding the lambda lysis genes, pPK20 (Fig. 2). This result suggests that LtgB degrades PG and may function as an autolysin.

The similarity of LtgB to MltC suggests that LtgB, like MltC, is a PG transglycosylase, i.e. it is predicted to cleave the N-acetylmuramic acid–β-1,4-N-acetylglucosamine bond and create a 1,6-anhydro bond on the N-acetylmuramic acid (Dijkstra & Keck, 1996). Residues important in this reaction have been characterized in the *E. coli* lytic transglycosylases Slt70 and Slt35 and in the lambda lytic transglycosylase R (Jespers et al., 1992; Leung et al., 2001; Thunnissen et al., 1994, 1995; van Asselt et al., 1999a, b, 2000). In order to examine the function of LtgB as a putative PG transglycosylase we made a point mutation in the putative active site of LtgB. The PG transglycosylases contain a conserved acidic residue necessary for enzymic activity. The family 1 PG transglycosylases, which include MltC, contain an invariant glutamic acid positioned adjacent to an invariant serine (Blackburn & Clarke, 2001). We identified a putative catalytic glutamic acid in LtgB by alignment with MltC (Fig. 1). A point mutation was made at this residue, changing glutamic acid 117 to alanine (*ltgB E117A*).

E. coli carrying the resulting plasmid, pPK36, did not lyse upon induction with arabinose (Fig. 2). A second ES motif is present at residues 99–100 of the putative LtgB amino acid sequence. Therefore we also made a point mutation at glutamic acid 99 to determine if E99 might be the necessary glutamic acid for PG transglycosylase activity. When the gene encoding this mutated version of LtgB (*ltgB E99A*) was substituted for *R* in the lambda lysis system (pPK39), lysis of *E. coli* was observed upon induction with arabinose (data not shown). These results suggest that glutamic acid 115 is necessary for the PG degradation activity of LtgB, and bring into question...
whether LtgB truly functions as a PG transglycosylase or may function as a peptidoglycanase with a different specificity.

**Creation of a point mutation in LtgB in the gonococcal chromosome using an insertional plasmid and positive and negative selection**

We have sought to create marker-less mutations in the lytic transglycosylase genes as we are attempting to make gonococcal mutants with multiple disruptions. In our mutagenesis of LtgA, we used a cassette containing *ermC*, an Erm resistance marker, and *rpsL*, a Str sensitivity marker, to interrupt LtgA in the gonococcal chromosome (Cloud & Dillard, 2002). The insertion was subsequently replaced in a second transformation using a plasmid carrying an *in vitro*-constructed deletion, a method developed for gonococci by Johnston & Cannon (1999). We devised a shortened method for introduction of a point mutation into LtgB (Fig. 3). A plasmid containing LtgB_E115N was constructed using pKCI, the plasmid carrying *ermC* and *rpsL*, as the parent vector. The resulting plasmid, pPK44, was transformed into N. gonorrhoeae MS11, and ErmR StrS transformants were obtained. Strain MS11, like many other gonococcal strains, is naturally StrS. However, Str sensitivity is dominant over Str resistance when both alleles of the gene are expressed (Lederberg, 1951). In order to select for isolates that had lost the inserted plasmid, ErmR transformants were grown without antibiotics in liquid culture and plated to medium containing Str. These isolates were replica-plated to medium containing Str or Erm to confirm Str resistance and to test for Erm sensitivity. Of 20 StrR ErmR isolates screened for the mutation by PCR and restriction digestion, 18 had retained the point mutation. The resulting gonococcal strain encoding LtgB_E115N was designated PK106.

Several elements of the mutagenesis were surprising. The frequency of StrR isolates was much higher than expected for resolution resulting in loss of the plasmid from the chromosome. In our previous experiments with inserted plasmids, we were unable to detect loss of the insertion in 10^7 cells (Hamilton et al., 2001). However, in the LtgB mutagenesis, StrR colonies were obtained at a frequency of 8.1 × 10^{-5} ± 2 × 10^{-7}. Also, Str resistance did not always indicate loss of the inserted plasmid. When StrR colonies were plated to medium containing Erm, 11.5% of StrR colonies were also ErmR, indicating that the original plasmid we had introduced, pPK44, was still inserted in the chromosome. To further investigate the appearance of StrR ErmR gonococci, we PCR amplified and sequenced *rpsL* from the inserted plasmid in four StrR ErmR isolates. Three of these isolates contained the same point mutation as is found in the original chromosomal copy of *rpsL*. The fourth contained a nonsense mutation in *rpsL*.

**Characterization of LtgB mutants of N. gonorrhoeae**

In order to examine the function of LtgB in *N. gonorrhoeae*, an in-frame deletion was created in LtgB. A plasmid containing the region surrounding LtgB and an in-frame deletion of the LtgB coding sequence was used to transform wild-type gonococcal strain MS11. We screened by PCR for homologous recombination and the loss of LtgB. The strain carrying the deletion of LtgB was designated KH539. We characterized this strain and PK106, carrying the LtgB_E115N mutation, for effects on PG fragment release.

To characterize PG turnover, gonococcal strains were grown in medium containing [6-3H]glucosamine and lacking glucose, to metabolically label the PG. Macromolecular PG was collected during a chase period and the amount of radioactivity present was determined to quantify the amount of the original PG that remained in the cell wall and the rate at which fragments were released into the culture supernatant. If LtgB contributes to PG fragment release, then the LtgB mutants would be expected to show a reduced rate of PG turnover. However, no significant difference in PG turnover was observed between the wild-type and mutant strains (Fig. 4).

To determine whether the same types of soluble PG fragments were being released during growth of the mutant and

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**Fig. 3.** Schematic of insertion-duplication, positive/negative selection mutagenesis. Plasmids containing *ermC* (Erm resistance), *rpsL* (Str sensitivity) and LtgB with the mutation of interest were transformed into MS11, and Erm^R^ colonies selected. Erm^R^ transformants were transferred to medium containing Str. Str^R^ colonies were screened for mutations in LtgB (represented by a star) by PCR and restriction endonuclease digestion.
The data presented here indicate that LtgB functions in PG degradation. LtgB was able to function in place of the lytic transglycosylase of the bacteriophage lambda lysis system. This activity and the sequence similarity with known lytic transglycosylases suggest that LtgB is a lytic transglycosylase, but further characterization of its biochemical activity would be necessary to demonstrate this function conclusively. Unfortunately, expression of ltgB in E. coli resulted in insoluble protein, and we were not able to assay the biochemical function of LtgB directly. Sequence analysis has shown that a glutamic acid directly adjacent to a serine is conserved among lytic transglycosylases (Blackburn & Clarke, 2001; Dijkstra & Thunnissen, 1994; Koonin & Rudd, 1994). This conserved glutamic acid is hypothesized to be the catalytic residue in lytic transglycosylases as it has been demonstrated to be for Slt70, Slt35 and lambda R (Jespers et al., 1992; Leung et al., 2001; Thunnissen et al., 1994, 1995; van Asselt et al., 1999a, b, 2000). The predicted amino acid sequence of LtgB shows a conserved glutamic acid residue at amino acid 117 adjacent to a serine. This group of residues aligns well with the predicted catalytic site of MltC from E. coli (Fig. 1) and the catalytic glutamic acid of E. coli Slt70. However, mutation of the putative active-site glutamic acid (E117) did not attenuate the enzymic activity of LtgB as measured by activity in the lambda lysis system. Further mutational analysis revealed that glutamic acid 115 (E115) is essential for LtgB function because a mutation changing E115 to an asparagine eliminated the ability of LtgB to lyse E. coli in the lambda lysis system. It is unclear how mutation of E115 might affect the function of LtgB. Replacement of E115 in LtgB with asparagine may not allow for proper conformation for substrate binding. Examination of the alignment of LtgB and MltC from E. coli (Fig. 1) reveals that in MltC there is a glutamine residue in the position occupied by E115 in LtgB. Thus, mutation of LtgB at E115 makes LtgB \(_{E115N}\) more similar to MltC at position 115 (position 216 in MltC) than wild-type LtgB. It is possible that E115 is the catalytic residue and, even though not in the expected position in the primary sequence, it may be in the correct position in the secondary structure to carry out the lytic transglycosylase reaction. Alternatively, this result may indicate that LtgB cleaves PG at a different bond from lytic transglycosylases and may have a different specificity from the related E. coli enzymes. Database searches using LtgB as the query showed that there are at least 17 more examples of lytic transglycosylase homologues with the ExES pattern that we observe in LtgB.

The studies presented here demonstrate the utility of combining insertion–duplication mutagenesis and positive and negative selection for the introduction of mutations into the gonococcal chromosome. Mutants were obtained at an appreciable frequency, and the undesired isolates that became Str sensitive but did not resolve out the duplication could be screened out by replica plating on medium containing Str and Erm. The reason for the high rate of Str\(^R\) Erm\(^R\) isolates is not completely clear, but we found two
different types of mutations in the introduced rpsL that resulted in StrR. Since three of the four mutations we found were identical to the sequence in the original StrR allele of rpsL, it appears that recombination between the resident rpsL (StrR) and the rpsL (StrR) on the inserted plasmid resulted in these mutations through a gene-conversion event. However, we also found a nonsense mutation in one of the StrR ErmR isolates. This result, together with the high frequency of ErmR StrR isolates, suggests that the production of both RpsL proteins may be detrimental. A similar phenomenon was observed when rpsL was used as a counter-selectable marker in Streptococcus pneumoniae (Sung et al., 2001).

Examination of PG turnover in the ltgB deletion mutant shows that LtgB does not significantly affect the amount of PG released. In addition, the ltgB deletion mutant still produced PG monomers in amounts different from the wild-type. Thus it is clear that LtgB does not substantially contribute to PG monomer production. This result raises the question of what the function of LtgB may be in N. gonorrhoeae. It is clear from E. coli lysis assays that LtgB can degrade PG. Thus it might function in the removal of PG strands during growth or division, or for breaking down fragments for PG recycling. However, we did not observe any defects in growth or division in the ltgB mutants, and we would expect mutants defective in recycling to show higher rates of PG turnover. A lack of phenotypes is also observed for E. coli lytic transglycosylase mutants. A triple lytic transglycosylase mutant in E. coli did not show any growth phenotypes (Lommatzsch et al., 1997), and a mutant lacking six lytic transglycosylases was only slightly affected in cell separation (Heidrich et al., 2002). It has been proposed that there is functional redundancy in PG hydrolases in E. coli, i.e. one of the many other PG-degrading enzymes may substitute for the lost function (Heidrich et al., 2002). This could be the case in N. gonorrhoeae as well. The common N. gonorrhoeae chromosome encodes five lytic transglycosylase homologues (Blackburn & Clarke, 2001; Cloud & Dillard, 2002, 2004) (GenBank accession AE004969), and the gonococcal genetic island present in most gonococcal strains encodes two additional lytic transglycosylase homologues (Dillard & Seifert, 1997; Hamilton et al., 2005). One of these enzymes or the PG-degrading amidase, endopeptidase or glucosaminidase (Chapman & Perkins, 1983; Hebeler & Young, 1976; Stefanova et al., 2003) of gonococci may substitute for LtgB function.

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