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Mutations in *ampG* or *ampD* Affect Peptidoglycan Fragment Release from *Neisseria gonorrhoeae*▽

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Neisseria gonorrhoeae **releases peptidoglycan fragments during growth. The majority of the fragments released are peptidoglycan monomers, molecules known to increase pathogenesis through the induction of proinflammatory cytokines and responsible for the killing of ciliated epithelial cells. In other gram-negative bacteria such as *Escherichia coli*, these peptidoglycan fragments are efficiently degraded and recycled. Peptidoglycan fragments enter the cytoplasm from the periplasm via the AmpG permease. The amidase AmpD degrades peptidoglycan monomers by removing the disaccharide from the peptide. The disaccharide and the peptide are further degraded and are then used for new peptidoglycan synthesis or general metabolism. We examined the possibility that peptidoglycan fragment release by *N. gonorrhoeae* results from defects in peptidoglycan recycling. The deletion of *ampG* caused a large increase in peptidoglycan monomer release. Analysis of cytoplasmic material showed peptidoglycan fragments as recycling intermediates in the wild-type strain but absent from the *ampG* mutant. An *ampD* deletion reduced the release of all peptidoglycan fragments and nearly eliminated the release of free disaccharide. The *ampD* mutant also showed a large buildup of peptidoglycan monomers in the cytoplasm. The introduction of an *ampG* mutation in the *ampD* background restored peptidoglycan fragment release, indicating that events in the cytoplasm (metabolic or transcriptional regulation) affect peptidoglycan fragment release. The *ampD* mutant showed increased metabolism of exogenously added free disaccharide derived from peptidoglycan. These results demonstrate that *N. gonorrhoeae* has an active peptidoglycan recycling pathway and can regulate peptidoglycan fragment metabolism, dependent on the intracellular concentration of peptidoglycan fragments.**

As *Neisseria gonorrhoeae* grows and divides, the cell wall is remodeled by peptidoglycanases that degrade intact peptidoglycan (PG) into smaller fragments (16, 23, 43). The principal PG fragments released from *N. gonorrhoeae* during growth are PG monomers, the basic subunits of the bacterial cell wall. These PG monomers are released in the anhydro form (1,6-anhydro disaccharide tetrapeptide and 1,6-anhydro disaccharide tripeptide) and are liberated by the action of lytic transglycosylases (7, 25, 43, 47). Purified anhydro PG monomers from *N. gonorrhoeae* reproduce the tissue damage of gonococcal infections, causing ciliated cell death when applied to human fallopian tubes in the organ culture model of pelvic inflammatory disease (37). The 1,6-anhydro disaccharide tetrapeptide monomers are identical to the tracheal cytotoxin of *Bordetella pertussis* and have been shown to induce the production of inflammatory cytokines interleukin-1 (IL-1) and IL-6 in human monocytes (8, 10, 24, 34, 45). The 1,6-anhydro disaccharide tripeptide monomers are identical to the PG fragments shown to stimulate Nod1 and lead to IL-8 production (4, 12, 13).

Gram-negative bacteria have evolved mechanisms for recycling PG fragments generated during normal growth (27, 42). PG monomers are translocated back into the cytoplasm via the AmpG transporter (6, 27, 32). Once inside the cytoplasm, these anhydro PG monomers are degraded, yielding glucosamine, alanine, and stem tripeptides. AmpD is a cytoplasmic *N*-acetylmuramyl-L-alanine amidase that is specific to anhydromuropeptides and acts to remove the stem peptide from the sugar residues (26, 28). LdcA is an L,D-carboxypeptidase that removes terminal D-Ala residues from stem tetrapeptides to form tripeptides (49). Murein tripeptides are then reincorporated into the PG biosynthetic pathway for the production of pentapeptide monomers to be incorporated back into the growing cell wall (15, 38). NagZ, a beta-*N*-acetylglucosaminidase removes *N*-acetylglucosamine from PG monomers or free disaccharide (5, 54, 55). Anhydromuramic acid kinase (AnmK) and MurQ, an *N*-acetylmuramic acid-6-phosphate etherase (51, 52), convert liberated *N*-acetylmuramic acid to *N*-acetylglucosamine phosphate.

Both *Escherichia coli* and gonococci break down up to 50% of their PG each generation (15, 23). However, because the PG fragments liberated by *E. coli* are efficiently recycled, only 3 to 10% of the PG fragments are released into the environment per generation (17). When they compared the PG turnover rate of *E. coli* with that of gonococci, Greenway and Perkins observed similar rates (9 to 15%) for gonococci and *E. coli* (18). Unlike *E. coli*, however, gonococci release immunologically relevant amounts of PG monomers into the extracellular milieu (36, 37). Significant release of PG monomers has been observed for other gram-negative bacterial pathogens including *B. pertussis* and *Haemophilus influenzae* (1, 2, 14). The question why these pathogens release greater amounts of PG fragments than *E. coli* remains unanswered. In *B. pertussis*, it

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TABLE 1. Strains and plasmids used in this study

Plasmid or strain	Description	Reference or source
Plasmids		
pIDN3	*N. gonorrhoeae* insertion-duplication plasmid (Erm^r)	22
pKH35	Gonococcal complementation vector (Cam^r) derived from pGCC6	21
pKH37	Gonococcal complementation vector (Cam^r) derived from pGCC6	30
pDG017	1,341-bp fragment containing *amiC* in pKH37 (Cam^r); *amiC* under *lac* promoter control	
pDG131	677-bp 5′ *ampG* fragment in pIDN3 (Erm^r)	This work
pDG132	*ampG* in-frame deletion construct; 722-bp 3′ *ampG* fragment in pDG131 (Erm^r)	This work
pDG133	*ampG* complementation; 1,331-bp fragment containing *ampG* of *N. gonorrhoeae* under *lac* promoter control in pKH37 (Cam^r)	This work
pDG140	1,892-bp *ampD* region in pIDN3 (Erm^r)	This work
pDG141	*ampD* in-frame deletion constructed in pDG140; contains 504-bp deletion internal to *ampD*	This work
pDG143	*ampD* complementation; 680-bp fragment containing *ampD* under *lac* promoter control in pKH37 (Cam^r)	This work
Strains		
MS11	Wild-type *N. gonorrhoeae* (Str^r)	48
DG132	MS11 transformed with pDG132; *ampG* deletion mutant	This work
DG133	MS11 transformed with pDG133; *ampG* complemented (Cam^r)	This work
DG142	MS11 transformed with pDG141; *ampD* deletion mutant	This work
DG143	DG142 transformed with pDG143; *ampD* complemented (Cam^r)	This work
DG147	DG142 transformed with pDG017; *amiC* overexpression	This work
DG152	DG132 transformed with pDG141; *ampD ampG* deletion mutant	This work

was shown that the expression of *E. coli ampG* greatly reduces PG monomer release (39). This result suggested that gram-negative bacteria that release PG monomers may do so because of defects in PG fragment uptake and recycling.

To examine PG recycling in *N. gonorrhoeae*, we made mutations in *ampG* and *ampD* and asked whether these mutants showed increased PG fragment release. Our results show that gonococcal AmpG is functional and proficient for the uptake of anhydro PG fragment monomers and free disaccharides. An *ampD* mutant showed decreased PG fragment release, a buildup of PG monomers in the cytoplasm, and an increase in the metabolism of imported PG fragments. These results demonstrate a functional PG recycling system in *N. gonorrhoeae* and suggest that the concentration of PG monomers within the cytoplasm is sensed by the cell for the regulation of PG metabolism.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Table 1 contains a list of all *N. gonorrhoeae* strains used in this study. Piliated gonococcal variants of strain MS11 or its derivatives were used for transformation, whereas nonpiliated variants were used for all other procedures. *N. gonorrhoeae* strains were grown with aeration in gonococcal base liquid medium (GCBL) containing Kellogg's supplements (29) and 0.042% $NaHCO_3$ (40) or on GCB agar plates (Difco) with Kellogg's supplements and 5% CO_2 (9). *E. coli* was grown in Luria broth or on Luria agar plates (46). Antibiotics erythromycin and chloramphenicol were both used at a concentration of 10 μg/ml for *N. gonorrhoeae*, and erythromycin was used at 500 μg/ml and chloramphenicol at 25 μg/ml for *E. coli*.

Plasmid construction. Plasmids used in this study are listed in Table 1. The primers, which include added restriction enzyme recognition sites (underlined below), were designed based on the *N. gonorrhoeae* strain FA1090 genome sequence (GenBank accession no. AE004969), unless indicated otherwise.

For amplification of *ampD* and the surrounding region, the primers 5′ ACA TCAAGCTTTGTAGGCGGTTTGGTAAATCTGCA 3′ and 5′ TTCTACTAG TGCTCGACCTTCGACAGCTCTTT 3′ were used with MS11 chromosomal DNA as the template. The amplicon contained the region from 614 bp upstream to 683 bp downstream of the *ampD* coding sequence. The PCR product was digested with HindIII and SpeI, ligated into the plasmid pIDN3 (22) to produce pDG140, and transformed into competent *E. coli* TAM1 cells. Plasmids from the resulting Erm^r transformants were screened for the predicted size and restriction digest patterns. To make a deletion in *ampD*, pDG140 was amplified with divergent primers containing BsaI restriction sites, 5′ GTCATAGGTCTCCAG ACGTGATTGTCCATGATGTTCTTCCTGTC 3′ and 5′GTCATAGGTCTCC GTCTTCGACTGGCGGCGGATA3′. The resulting PCR product was digested with BsaI, self-ligated, and transformed into *E. coli*. Plasmids from the resulting Erm^r transformants were screened for the predicted size and for the expected restriction digest patterns. One of the plasmids with the correct predicted size was confirmed to contain the expected deletion by DNA sequencing and was named pDG141.

For complementation, the *ampD* gene lacking its predicted intrinsic promoter was PCR amplified from MS11 by using the primers 5′ TTCTACTAGT CGCC GGACAGGAAGAACATC 3′ (SpeI) and 5′ ACATCAAGCTTGCAGGGTCT GACAGCAGTGT 3′ (HindIII). The resulting 680-bp product was cut and ligated into pKH37 at the SpeI and HindIII restriction sites to generate pDG143. Transformants containing pDG143 were selected for chloramphenicol resistance. Correct plasmid construction was verified by PCR amplification and restriction digest mapping.

To construct the *ampG* deletion mutant, both the 5′ and the 3′ ends of *ampG*, including their flanking sequences, were PCR amplified separately using two primer pairs containing restriction sites. The 677-bp fragment containing the 5′ portion (8 bp) of the *ampG* sequence was amplified with primers 5′ ACATCA AGCTTAACACGCCAAAGCCCTGAAC 3′ (HindIII) and 5′ AGTAGG ATCC GCAGTCATCGTTCGTACAGCCAA 3′ (BamHI). The product was cut and ligated into pIDN3 at the HindIII and BamHI restriction sites to generate pDG131. The 722-bp fragment containing the 3′ portion (79 bp) of *ampG* was amplified with the primers 5′ TTCTACTAGTCGGCGAGGGCTGTACC GTAT 3′ (SpeI) and 5′ AGTAGGATCCACTTGCCCTGCCGGGTATG 3′ (BamHI). The product was cut and ligated into pDG131 at the SpeI and BamHI restriction sites to generate pDG132. Erm^r transformants were screened for plasmids of the predicted size and the expected restriction digest patterns.

For complementation using *ampG*, the gene lacking its predicted promoter was PCR amplified from strain MS11 chromosomal DNA by using the primers 5′ TTCTACTAGT TGGCTGTACGAACGATGACTGCA 3′ (SpeI) and 5′ ACA TCAAGCTT CAATATCAGGTAAACGCTCCAGTTTGA 3′ (HindIII). The resulting 1,340-bp product was cut and ligated into pKH37 at the SpeI and HindIII restriction sites to generate pDG133. Transformants containing pDG133 were selected for chloramphenicol resistance. Correct plasmid construction was verified by PCR amplification, restriction digest mapping, and DNA sequencing.

***ampG* and *ampD* mutants of *N. gonorrhoeae*.** To construct a deletion mutation in *ampD*, the wild-type gonococcal strain MS11 was transformed with NheI-linearized pDG141. Transformants were screened for the absence of *ampD* by PCR, using both intragenic and *ampD*-flanking primer pairs to identify an *ampD*

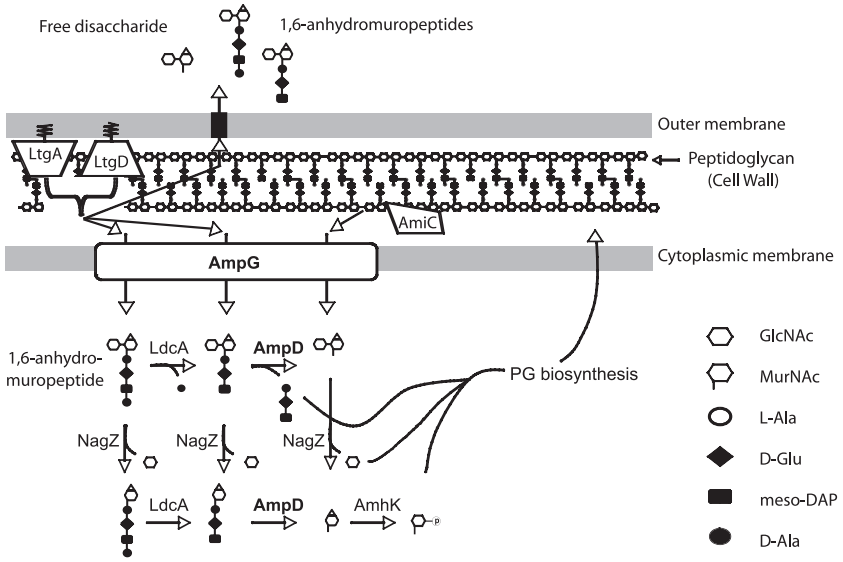

FIG. 1. Model of peptidoglycan recycling in *Neisseria gonorrhoeae*. As the bacterial cell grows and divides, the sacculus is remodeled by peptidoglycanases including lytic transglycosylases LtgA and LtgD and amidase AmiC. The liberated PG fragments include 1,6-anhydro PG monomers and free disaccharide. The gonococcal genome was found to encode homologues of multiple PG recycling proteins, and their predicted functions are shown here. PG fragments are taken into the cytoplasm through the AmpG permease to be further degraded by a carboxypeptidase (LdcA), an *N*-acetylmuramyl-L-alanine amidase (AmpD), and an *N*-acetylglucosaminidase (NagZ). The order in which these reactions occur in gonococci is not known. The resulting tripeptide is incorporated within the PG biosynthetic pathway leading to UDP-pentapeptide monomers for incorporation into the growing sacculus. The symbols used to represent amino acids and sugar residues were chosen to mirror those used by Jacobs et al. (27).

deletion mutant (DG142). Complementation of the *ampD* mutant was generated by transformation with pDG143 containing a gonococcal *ampD* allele under the control of the *lac* promoter-operator. Plasmid pDG143 contains portions of the gonococcal *aspC* and *lctP* genes that facilitate insertion of the complementation construct between these genes in the chromosome (Table 1). Complemented *ampD* strains were selected for their resistance to chloramphenicol and were screened by PCR for incorporation of the additional *ampD* allele and retention of the *ampD* deletion mutation.

To create a deletion mutation in *ampG*, pDG132 was linearized with NheI and introduced into *N. gonorrhoeae* by spot transformation (20). Potential transformants were screened for the absence of *ampG* by PCR, using intragenic and *ampG*-flanking primers. DNA sequencing confirmed the expected deletion. Complementation of the *ampG* mutant DG132, was performed by spot transformation using plasmid pDG133. After transformants were selected with chloramphenicol, they were screened by PCR, using primers to verify retention of the *ampG* deletion and insertion of the *ampG* allele at the region of homology. Sequencing of the introduced *ampG* allele confirmed the expected sequence.

For the creation of an *ampD ampG* double mutant, strain DG132 was transformed with pDG141 linearized with NheI. Transformants were screened for a deletion in *ampD* by PCR using both intragenic and *ampD*-flanking primer sets. To express *amiC* in an *ampD* background, strain DG142 was transformed using pDG017, a plasmid that contains *amiC* under the control of the *lac* promoter (11). Transformants resistant to chloramphenicol were screened by PCR using primers specific to *amiC* and flanking the chromosomal genes *aspC* and *lctP*.

Characterization of released PG fragments. Labeling, purification, and characterization of gonococcal PG were performed after the method described by Rosenthal and Dziarski (44), with modifications described by Cloud and Dillard (7). Gonococcal strains were pulse-labeled by growth in GCB medium containing [6-^{3}H]glucosamine and lacking glucose. To allow for quantitative comparisons, the amount of radioactivity in the cells was determined for each culture, after the labeling period, and the cultures were diluted to achieve equivalent amounts of radioactivity in the cells for the chase period. PG fragments released into the medium during the chase period were separated by size-exclusion chromatography and detected by liquid scintillation counting. The relative amounts of PG monomers released were quantified by determining the area under the curve for the PG monomer peaks, using the method described by Vossoughi (53). High-performance liquid chromatography (HPLC) analysis was carried out as described by Kohler et al. (30). Fractions from the size-exclusion column containing PG monomers were pooled, desalted, and lyophilized. PG monomers were separated by C_{18} reversed-phase HPLC using a 4 to 13% acetonitrile gradient for 30 min.

Preparation of hot-water extracts. Cell-free hot-water extracts were prepared as described by Jacobs et al. (27). Each sample represented the hot-water extract from approximately 3×10^9 logarithmic phase cells. Gonococci were grown with aeration for 35 min in GCBL medium lacking glucose and containing 0.4% pyruvate plus [6-^{3}H]glucosamine at a concentration of 10 μCi/ml to label the PG. After samples were centrifuged for 5 min at 1,800 × *g*, supernatants were discarded, and cells were washed with GCBL and resuspended in 6 ml of GCBL with glucose and without label. Triplicate 100-μl samples were taken from each culture to measure cpm, to normalize the amount of label incorporated into the cells. Cultures were grown with aeration for 45 min, quickly chilled to 4°C, centrifuged at 1,800 × *g*, and washed once with 1.5 ml of cold water. Cells were then suspended in 2 ml of boiling water and maintained at 100°C for 5 min. To remove particulate matter, samples were centrifuged at 12,000 × *g* for 10 min at 4°C, and supernatants (hot-water extracts) were collected. For direct comparison, the amount of sample loaded onto the column was adjusted to account for the total amount of label incorporated by each individual culture.

Uptake of purified PG fragments. Metabolically labeled PG monomers and free disaccharide were purified from MS11 culture supernatant by size-exclusion chromatography, as described above. Purified PG monomers (4.8×10^6 cpm) or free disaccharide (1.6×10^6 cpm) suspended in 400 μl water were added to 3 ml wild-type or mutant gonococcal strains growing in log-phase culture. The amount of labeled PG monomers or free disaccharide was equivalent to 60% of that found in log-phase culture. After 1 h of growth, cells were harvested by centrifugation for 10 min at 1,800 × *g*. Radioactivity levels in the cell pellets were determined by liquid scintillation counting and were normalized to total protein content. Protein concentrations in cell pellets were determined by the Lowry method using the Bio-Rad D_C protein assay as outlined in the manufacturer's instructions (33).

RESULTS

Peptidoglycan recycling genes in *N. gonorrhoeae*. Although *N. gonorrhoeae* releases PG fragments during growth, the genes for PG recycling proteins are found in the *N. gonorrhoeae* genome (GenBank accession no. AE004969) (Fig. 1).

Homologues were found for AmpG (NGO1599), a permease that acts to transport PG fragments across the inner membrane (6, 32); for LdcA (NGO1274), a carboxypeptidase that removes the terminal alanine from PG tetrapeptide monomers (49); for AmpD (NGO0237), an amidase that splits PG monomers into disaccharide and peptide portions (26); and for NagZ (NGO0135), a beta-glucosaminidase that removes the *N*-acetylglucosamine from PG monomers or free disaccharide (5, 54, 55). A homologue for AnmK was also found (NGO1583). AnmK acts to phosphorylate anhydro-*N*-acetylmuramic acid and eliminates the 1,6-anhydro bond (52). However, no homologue was found for MurQ, which in *E. coli*, acts to convert *N*-acetylmuramic acid-phosphate to *N*-acetylglucosamine-phosphate (51).

The gonococcal AmpG coding sequence is significantly shorter than that of *E. coli*. An amino acid alignment of the gonococcal and *E. coli* AmpG proteins shows gonococcal AmpG is shorter at the C-terminal end by 77 amino acids, approximately 15% of the coding sequence. Chahboune et al. proposed a topological model of *E. coli* AmpG inner membrane protein showing 10 membrane-spanning regions (3). A topological profile of the predicted membrane-spanning regions for gonococcal and *E. coli* AmpG protein homologues shows the former lacking two membranes, one periplasmic and two cytoplasmic regions at the C-terminal end. It is also notable that the helical regions 5 and 6 of gonococcal AmpG have lower prediction probabilities for membrane spanning and are assigned a probable and transient residence in the periplasm or cytosol (data not shown). These results suggested that the gonococcal AmpG might not function or might not function as well as *E. coli* AmpG.

Mutation of *ampG* increases the release of PG monomers. To test the functionality of AmpG and its role in PG recycling, we constructed an in-frame deletion of *ampG* in the gonococcal chromosome and characterized PG fragments released by the *ampG* mutant. During growth, *N. gonorrhoeae* sheds soluble PG fragments including PG multimers, PG monomers, and free disaccharide (43, 47). PG monomers are the predominant soluble fragments found in gonococcal culture supernatants and are the molecules that cause inflammatory cytokine induction and the death of human ciliated cells (10, 37, 47).

To measure the amount of PG fragments released into the supernatant during growth, each gonococcal strain was pulse-labeled metabolically by growing cells in medium containing [6-^{3}H]glucosamine. Cultures containing equivalent amounts of radiolabel were grown for 2.5 h in medium without label to allow release of PG fragments, and the released fragments were characterized by size-exclusion chromatography. The resulting profile of the *ampG* deletion mutant (DG132) showed an increase in PG monomer release of approximately sevenfold compared to that of the wild type (Fig. 2). Complementation of the *ampG* deletion mutant with wild-type *ampG* at a distant location on the gonococcal chromosome (DG133) restored wild-type levels of PG monomer release.

To determine if AmpG acts in the transport of PG fragments across the inner membrane, PG fragments in the cytoplasm of the wild-type or the *ampG* mutant strain were prepared by hot water extraction, as described by Jacobs et al. (27). Gonococci were pulse-labeled in medium containing [6-^{3}H]glucosamine and lacking glucose. Cells were washed and resuspended in medium containing glucose and allowed to grow for an additional 45 min. Cells were subjected to osmotic shock by washing them with water to remove periplasmic contents. The cells were then boiled in water to obtain the hot-water extracts. The soluble cytosolic material was then separated by size-exclusion chromatography, and PG fragments were detected by scintillation counting. The wild-type strain MS11 showed a peak slightly larger than that of the PG monomer standard (Fig. 3A). This material likely represents the UDP-*N*-acetylmuramic acid (MurNAc) pentapeptide, the PG biosynthesis precursor, which is the only detectable peak in similar hot-water extracts of *E. coli* (27). By contrast, the *ampG* deletion mutant showed no cytoplasmic PG fragments (Fig. 3A). This result demonstrates that AmpG is required for the uptake of PG fragments into the cytoplasm. Overall, these results suggest that gonococcal AmpG is a functional PG fragment permease and indicate that *N. gonorrhoeae* recycles approximately 85% of liberated PG monomers generated during growth in vitro.

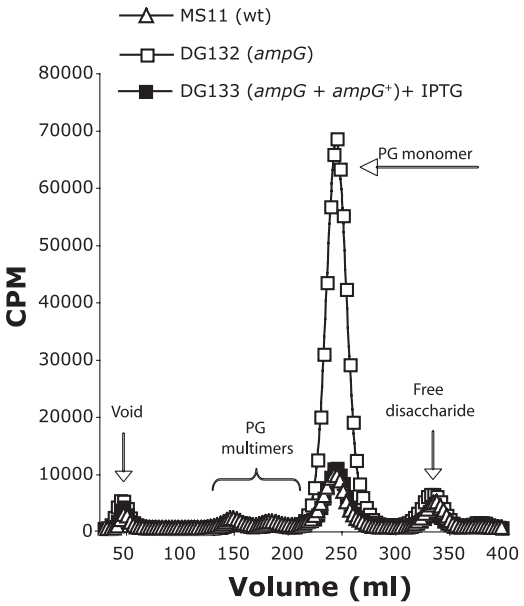

FIG. 2. Mutation of *ampG* causes an increase in PG monomer release. *N. gonorrhoeae* strains MS11 (wild type), DG132 (*ampG*), and DG133 (*ampG* + *ampG*$^+$, complemented strain) were labeled metabolically by growth in glucose-deficient medium containing [6-^{3}H]glucosamine. Supernatants were collected after 2.5 h of growth, and released PG fragments were separated by size-exclusion chromatography and detected by scintillation counting.

Mutation of *ampD* diminishes PG fragment release. AmpD in *E. coli* is a cytoplasmic *N*-acetylmuramyl-L-alanine amidase that specifically recognizes and cleaves anhydro PG monomers (26, 28). We made an *ampD* deletion mutant so that we could assess effects that the lack of PG recycling might have on growth characteristics. It has been speculated that the purpose of PG recycling is to recover nutrients. However, measurements of growth in culture showed that neither the *ampG* mutant nor the *ampD* mutant was deficient in growth under these conditions (data not shown).

We measured PG fragment release from the *ampD* mutant and from the wild-type strain. The *ampD* deletion mutant (DG142) exhibited a decrease in the release of all small PG fragments including a 40% reduction in PG monomers and a

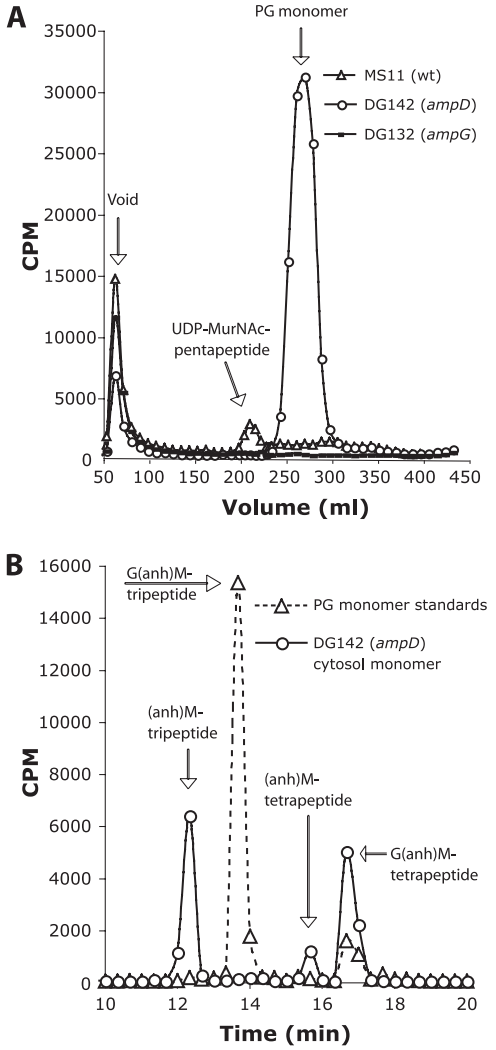

FIG. 3. Hot-water extracts to detect PG fragments in the cytoplasm. (A) The PG of strains MS11 (wild type), DG132 (*ampG*), and DG142 (*ampD*) were labeled by growth in medium containing [6-^{3}H]glucosamine. After a 45-min chase period, cytoplasmic extracts were prepared, and the material was separated by size-exclusion chromatography. The PG monomer peak shown in panel A was analyzed by C_{18} reversed-phase HPLC (B). The dotted line shows the retention times of PG monomer standards. Identities of the *ampD* cytosolic monomers are predicted based on their elution from the sizing column and retention times on the C_{18} column. anh, anhydro; G, *N*-acetylglucosamine; M, *N*-acetylmuramic acid.

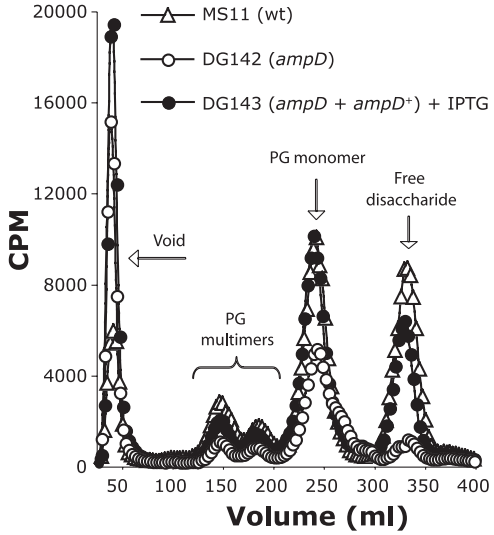

FIG. 4. An *ampD* deletion mutant exhibits a decrease in the release of PG multimers, PG monomers, and free disaccharide. *N. gonorrhoeae* strains MS11 (wild type), DG142 (*ampD*), and DG143 (*ampD* + *ampD*$^+$, complemented strain) were labeled by growth in glucose-deficient medium containing [6-^{3}H]glucosamine. Supernatants containing radiolabeled PG fragments were collected after 2.5 h of growth and passed over tandem size-exclusion columns. Fractions containing radiolabeled PG fragments were detected by scintillation counting.

dramatic reduction in free disaccharide levels compared to the wild type (Fig. 4). Also, an additional species appeared in the *ampD* profile as an unresolved peak with a slightly smaller molecular weight compared to that of PG monomers. We speculate that this peak may represent PG monomers from which the *N*-acetylglucosamine has been cleaved. Complementation with a wild-type copy of *ampD* at another location on the chromosome restored the wild-type phenotype.

Examination of the cytoplasmic PG fragments by using hot-water extracts showed that the *ampD* mutant accumulated PG monomers (Fig. 3A). The *ampD* mutant was predicted to build up 1,6-anhydro-*N*-acetylmuramic acid (anhMurNAc) tripeptide monomers, since the mutant should not be able to degrade imported PG monomers further than the removal of GlcNAc and the terminal alanine (Fig. 1). Reversed-phase HPLC separation of the monomer peak from the *ampD* hot-water extracts showed that this material was composed of three separate monomers (Fig. 3B). The major peak is the expected anhMurNAc tripeptide. A minor peak is likely the related anhMurNAc tetrapeptide. A third peak was identified, with the same retention time as GlcNAc-anhMurNAc tetrapeptide. The absence of the PG biosynthesis precursor seen in the wild-type profile and the accumulation of PG monomers in the cytoplasm of the *ampD* mutant demonstrate that AmpD is required for the breakdown and reuse of imported PG monomers.

Mutation of *ampD* results in increased metabolism of PG fragments. The large reduction in free disaccharide release by the *ampD* mutant (Fig. 4) was surprising since we had previously found that a different amidase, AmiC, is responsible for the generation of free disaccharide in *N. gonorrhoeae*. In the *amiC* mutant, free disaccharide release was completely abolished (11). The generation of free disaccharide should occur in the periplasm by the combined actions of a lytic transglycosylase and an amidase. Free disaccharide would then diffuse across the outer membrane. Why then should a mutation affecting a cytoplasmic amidase affect the generation or release of a PG fragment in the periplasm? We considered three possibilities to explain these results. (i) AmiC might be inhibited or produced in lower amounts in the absence of *ampD*. (ii) AmpD might be responsible for the production of free disaccharide. (iii) A buildup of PG monomers in the cytoplasm might affect the transcription of genes for PG fragment deg-

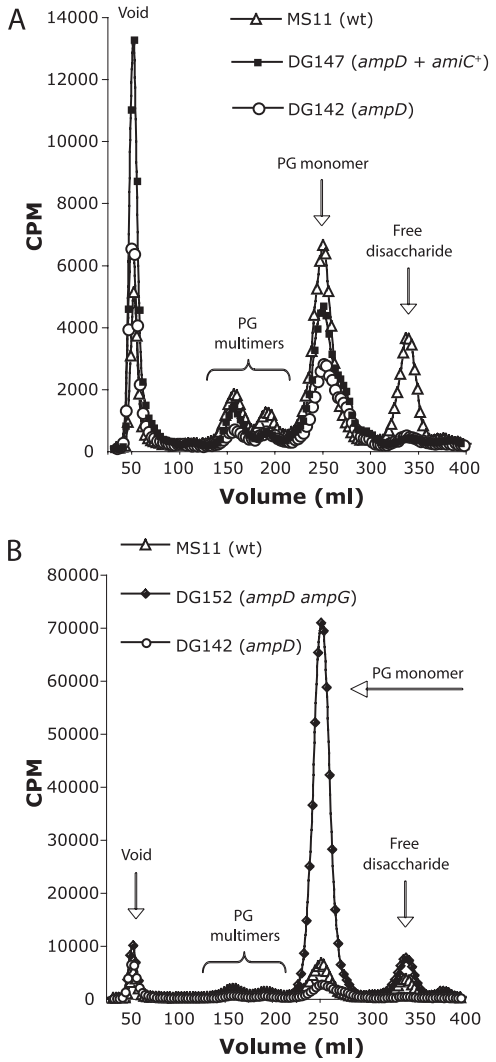

FIG. 5. Free disaccharide release by the *ampD* mutant is not restored by the expression of *amiC* but is restored by the mutation of *ampG*. PG fragments released from strains MS11 (wild type), DG142 (*ampD*), and DG147 (*ampD* + *amiC*$^+$) induced with IPTG and from the strain DG152 (*ampD ampG*) were separated by size and detected by scintillation counting. The expression of *amiC* does not significantly alter the release of PG fragments from the *ampD* mutant (A). Mutation of *ampG* restores the amount of free disaccharide released by the *ampD* mutant to levels that are slightly higher than those observed with the wild-type strain (B).

radation, or it might affect the activity of enzymes involved in PG fragment breakdown.

If the amounts of AmiC were reduced or if the activity of AmiC were inhibited in the *ampD* mutant, then the overexpression of *amiC* might restore free disaccharide production. Therefore, we transformed the *ampD* mutant strain DG142 with a construct that carries *amiC* under the control of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *lac* promoter-operator pDG017 (11). PG fragment release was not restored by the overexpression of *amiC* (Fig. 5A), suggesting that the loss of free disaccharide release was not due to effects on AmiC.

By introducing an *ampG* mutation into the *ampD* background, we were able to separate events occurring in the periplasm that affect PG breakdown from those that occur in the cytoplasm. An *ampG* mutant should be unable to transport PG monomers or free disaccharide into the cytoplasm, and thus no buildup of PG monomers should occur in the double mutant. The PG release profile for the *ampG ampD* double mutant showed that free disaccharide release was restored to wild-type levels (Fig. 5B). This result shows that *ampD* is not required for free disaccharide production. This result also demonstrates that transport of PG fragments into the cytoplasm is necessary for the reduced release of PG fragments seen with the *ampD* mutant.

The decreased release of free disaccharide by the *ampD* mutant suggested that this mutant was metabolizing more of the PG fragments that were generated by PG breakdown; i.e., free disaccharide from PG degradation was not being released at wild-type amounts because these PG fragments were being transported into the cytoplasm, broken down into *N*-acetylglucosamine and *N*-acetylmuramic acid, and incorporated into non-cell wall material to a greater extent than in the wild-type strain. To test this hypothesis, we added purified radiolabeled PG fragments to gonococci and measured the incorporation of the labeled PG material into the cells. Labeled free disaccharide or PG monomers were purified and added to the wild-type, *ampD*, or *ampG* gonococci growing in log-phase culture. Free disaccharide was incorporated to a greater extent by the *ampD* strain than by the wild-type strain, confirming increased PG fragment metabolism in the *ampD* mutant (Fig. 6A). PG monomers were incorporated in equal amounts by the wild-type and *ampD* strains (Fig. 6B). The *ampG* mutant showed minimal incorporation of free disaccharide or PG monomers, consistent with the requirement for AmpG for transport of anhydro-PG fragments into the cytoplasm (6). The results shown in Fig. 6A should not be compared to those shown in Fig. 6B to evaluate the relative ability of AmpG to transport PG monomers versus free disaccharide, because different amounts of radioactivity were used in the two experiments and because the abilities of PG monomers and free disaccharide to cross the outer membrane are likely different.

Together these results suggest a model in which AmpG acts to transport PG fragments across the cytoplasmic membrane, and AmpD breaks down the monomers to peptides and disaccharide that are preferentially incorporated into new cell wall synthesis. In the *ampD* mutant, a buildup of PG monomers causes increased metabolism of PG fragments, and *N*-acetylglucosamine released from PG monomers or *N*-acetylglucosamine and *N*-acetylmuramic acid derived from free disaccharide, is incorporated into PG and non-PG cell material.

DISCUSSION

It has been speculated that the reason that *N. gonorrhoeae* sheds PG monomers into the extracellular milieu during growth is because it is deficient in PG fragment uptake and recycling. However, we have shown that gonococci have a functional PG recycling system. Homologues of five PG fragment-recycling proteins were found encoded in the gonococcal genome. No homologue was found for MurQ, which in *E. coli*, acts to convert MurNAc-phosphate to GlcNAc-phosphate (51). *N. gonorrhoeae* may not perform the MurQ function and

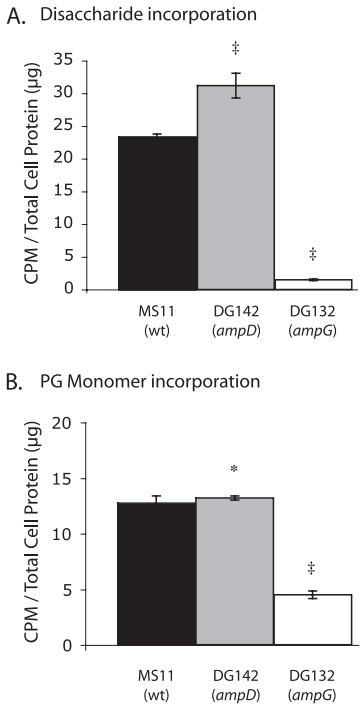

FIG. 6. The *ampD* mutant incorporates more radiolabeled free disaccharide (A) than the wild type. The *ampG* mutant is deficient in the uptake of both PG monomers (B) and free anhydro disaccharides (A). The *N. gonorrhoeae* MS11 (wild-type), DG142 (*ampD*), and DG132 (*ampG*) strains were labeled by growth in medium containing glucose and purified PG fragments for 1 h. Cells were centrifuged and resuspended in water, and radioactivity was measured by scintillation counting to determine the level of incorporation of radiolabeled PG monomers, free disaccharide, or glucosamine (data not shown). Counts per minute were normalized for total cell protein. The values shown are the averages of three cultures per strain with at least three measurements from each culture. Error bars represent the standard deviations. ‡, Student's *t* test *P* value of <0.01 compared to that of the wild type; asterisk, not statistically different from that of the wild type.

may be able to use MurNAc-phosphate directly, or gonococci may use an unrelated enzyme for this purpose. The mutation of *ampG* resulted in a sevenfold increase in PG monomer release. This result suggests that gonococci normally recycle 85% of the PG monomers liberated by peptidoglycanases during growth and division.

The role of gonococcal AmpG in the uptake of PG fragments was confirmed by our results showing that exogenously added PG monomers or free disaccharide were taken up efficiently by the wild-type strain but were taken up poorly by the *ampG* mutant (Fig. 6). Given this result, it is somewhat surprising that the *ampG* mutant did not show a significant increase in the release of free disaccharide (Fig. 2). Instead, a very minor increase in free disaccharide loss is seen with the *ampG* mutant compared to that of the wild type, and a similar slight increase in free disaccharide loss is seen with the *ampG ampD* double mutant compared to that of the wild type (Fig. 5B). Two possible explanations may account for these results. One possibility is that gonococci may normally release most of the free disaccharide produced. The mutation of *ampG* would then affect only the small amount normally recycled. Perhaps gonococcal AmpG has increased specificity for PG monomers compared to that of free disaccharide. Such a specificity would make sense from an immunological viewpoint, since it could be advantageous for the bacteria to control the release of the proinflammatory PG monomers under some conditions, whereas controlling the release of the apparently immunologically inert free disaccharide would be of less importance. A second possibility is that gonococci have a second transporter for free disaccharide uptake, and it continues to operate in the *ampG* mutant. This possibility seems less likely, since the *ampG* mutant is dramatically reduced in free disaccharide uptake (Fig. 6A) and shows no PG fragments in the cytoplasm (Fig. 3A).

A deletion of *ampD* affected PG fragment recycling but not in the way we expected. The *ampD* mutants should be unable to cleave the bond between the *N*-acetylmuramic acid and the stem peptide in imported PG monomers and thus should be unable to use the *N*-acetylmuramic acid for cell wall biosynthesis or general metabolism. An *ampD* mutant might show increased release of PG monomers, since the mutant is unable to fully degrade these molecules. The appearance of an additional monomer peak of a smaller molecular size on the *ampD* fragment release profile is consistent with the buildup of anhydromuramic acid tripeptide monomers in the *ampD* mutant (Fig. 4). These fragments may be generated by NagZ activity in the cytoplasm and may be released by autolysis in a portion of the population. The surprising result was that the *ampD* mutant's release of free disaccharide was reduced (Fig. 4). In pulse-chase experiments, the liberated PG fragments were found in the cell as PG monomers in the cytoplasm and as other, non-PG cell constituents (Fig. 3). Since the labeled disaccharide was not released into the medium, we hypothesized that these PG fragments were being metabolized and used for the synthesis of other cell constituents at a higher rate than in the wild-type strain. This hypothesis was confirmed by the greater degree of incorporation of exogenously added free disaccharide by the *ampD* mutant (Fig. 6A). Thus, it appears that gonococci regulate PG fragment recycling in response to changes in cytoplasmic levels of PG monomer.

The mechanism for the increased utilization of PG free disaccharide fragments by the *ampD* mutant is unclear. It could be due to increased uptake of PG fragments or increased breakdown of the fragments in the cytoplasm. Increased uptake of disaccharide might occur by an increase in AmpG production or by an increase in a second, hypothetical, free disaccharide transporter. An increase in NagZ levels or function could result in increased levels of *N*-acetylglucosamine in the cytoplasm that could then be used for general metabolism or cell wall synthesis. It is known that *Citrobacter freundii* can respond to increased levels of PG monomers by transcriptional regulation. AmpR binds to PG monomers and derepresses the transcription of *ampC*, encoding a beta-lactamase (31). Gonococci, like many gram-negative bacterial species, do not encode an obvious AmpR orthologue. However, changes in PG fragment metabolism in the *ampD* mutant suggest that gonococci may have some other transcriptional regulator to control PG fragment metabolism.

Since it is now clear that gonococci have an efficient PG uptake and recycling system, then why do gonococci produce a PG-related inflammatory response and ciliated cell death, while bacteria such as *E. coli* do not? One possibility is that PG

fragment release is regulated during infection. It is clear from the results shown in Fig. 2 and Fig. 4 that AmpG and AmpD have significant effects on PG fragment release. By regulating the amounts of these proteins, gonococci could promote the inflammatory response or decrease it. Gonococci release multiple proinflammatory molecules including LOS (19), porin (35), DNA (21), and PG (47), suggesting that under some circumstances an inflammatory response favors the bacteria. Gonococci often cause asymptomatic infections in women (41), suggesting that the release of proinflammatory molecules such as PG may be reduced during those infections. A second possibility is that gonococci do not differ from *E. coli* in the overall amounts of PG fragments released but only in the types of fragments released. It was recently shown that *E. coli* produces a periplasmic amidase that specifically degrades PG monomers (50). This process would detoxify the liberated fragments (12, 34), and the released fragments would not provoke an immune response.

In summary, we have demonstrated a functional PG recycling pathway in *N. gonorrhoeae*, where gonococcal AmpG and AmpD proteins are responsible for the translocation and metabolism of PG fragments. Furthermore, we have demonstrated that an *ampD* mutant deficient in PG monomer degradation has increased metabolism of PG fragments and decreased release of free disaccharide from PG into the medium, suggesting that gonococci regulate PG fragment degradation based on PG fragment levels in the cytoplasm.

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