2009

Mutations in ampG and lytic transglycosylase genes affect the net release of peptidoglycan monomers from Vibrio fischeri

Dawn M. Adin  
*University of Georgia*

Jacquelyn T. Engle  
*Washington University School of Medicine in St. Louis*

William E. Goldman  
*Washington University School of Medicine in St. Louis*

Margaret J. McFall-Ngai  
*University of Wisconsin - Madison*

Eric V. Stabb  
*University of Georgia*

Follow this and additional works at: [https://digitalcommons.wustl.edu/open_access_pubs](https://digitalcommons.wustl.edu/open_access_pubs)

**Recommended Citation**
[https://digitalcommons.wustl.edu/open_access_pubs/2445](https://digitalcommons.wustl.edu/open_access_pubs/2445)
Mutations in ampG and Lytic Transglycosylase Genes Affect the Net Release of Peptidoglycan Monomers from Vibrio fischeri

Dawn M. Adin, Jacquelyn T. Engle, William E. Goldman, Margaret J. McFall-Ngai and Eric V. Stabb

Mutations in ampG and Lytic Transglycosylase Genes Affect the Net Release of Peptidoglycan Monomers from Vibrio fischeri

Dawn M. Adin, Jacquelyn T. Engle, William E. Goldman, Margaret J. McFall-Ngai, and Eric V. Stabb

Department of Microbiology, University of Georgia, Athens, Georgia 30605; Department of Molecular Microbiology, Washington University, St. Louis, Missouri 63110; Department of Microbiology and Immunology, University of North Carolina, Chapel Hill, North Carolina 27517; and Department of Medical Microbiology and Immunology, University of Wisconsin, Madison, Wisconsin 53706

Received 31 October 2008/Accepted 5 December 2008

The light-organ symbiont Vibrio fischeri releases N-acetylglucosaminyl-1,6-anhydro-N-acetylmuramylalanylglutamyldiaminopimelylalanine, a disaccharide-tetrapeptide component of peptidoglycan that is referred to here as “PG monomer.” In contrast, most gram-negative bacteria recycle PG monomer efficiently, and it does not accumulate extracellularly. PG monomer can stimulate normal light-organ morphogenesis in the host squid Euprymna scolopes, resulting in regression of ciliated appendages similar to that triggered by infection with V. fischeri. We examined whether the net release of PG monomers by V. fischeri resulted from lytic transglycosylase activity or from defects in AmpG, the permease through which PG monomers enter the cytoplasm for recycling. An ampG mutant displayed a 100-fold increase in net PG monomer release, indicating that AmpG is functional. The ampG mutation also conferred the uncharacteristic ability to induce light-organ morphogenesis even when placed in a nonmotile flaJ mutant that cannot infect the light-organ crypts. We targeted five potential lytic transglycosylase genes singly and in specific combinations to assess their role in PG monomer release. Combinations of mutations in ltgA, ltgD, and ltgY decreased net PG monomer release, and a triple mutant lacking all three of these genes had little to no accumulation of PG monomers in culture supernatants. This mutant colonized the host as well as the wild type did; however, the mutant-infected squid were more prone to later superinfection by a second V. fischeri strain. We propose that the lack of PG monomer release by this mutant results in less regression of the infection-promoting ciliated appendages, leading to this propensity for superinfection.

Microbe-associated molecular patterns (MAMPs) are recognized by hosts in a variety of pathogenic and symbiotic relationships. MAMP is an umbrella term for a variety of conserved bacterial molecules, including lipopolysaccharide, lipoproteins, flagella, and peptidoglycan (PG), that are sensed by conserved host surveillance mechanisms (e.g., the innate immune system), triggering context-dependent reactions to bacterial colonization. Mounting evidence shows that PG-derived MAMPs play important and previously underappreciated roles in host-bacterium interactions (11).

The PG layer of gram-negative bacteria is a rigid network in the periplasm that protects against osmotic lysis and helps to determine cell size and shape while still allowing diffusion of molecules into the cell (14). In PG, repeated subunits of N-acetylglucosamine and N-acetylmuramic acid are connected to a short pentapeptide side chain of L-alanyl-D-γ-glutamyl-meso-diaminopimelyl-D-alanine (Ala-Glu-DAP-Ala-Ala). Adjacent peptides are cross-linked through Ala-DAP or DAP-DAP peptide bonds, and side chains are converted to tetra-, tri-, and dipeptides through the action of carboxypeptidases in the periplasm (19, 49).

Despite its mechanical stability, PG is a dynamic structure that undergoes remodeling and recycling. Murein hydrolases, including lytic transglycosylases, hydrolyze PG to allow the insertion of new material as the cell grows or to accommodate structures that span the periplasm (61, 72). Lytic transglycosylases cleave the N-acetylmuramic acid-β-1,4-N-acetylglycosamine linkage in PG and catalyze the formation of a 1,6-anhydro bond on the N-acetylmuramic acid (28). After cleavage, PG monomers enter the cytoplasm through the permease AmpG (30) and are recycled and ultimately reincorporated into PG (20). Murein hydrolase activity and the recycling of PG monomers presumably allow for growth and cell expansion; however, Escherichia coli mutants lacking ampG or several lytic transglycosylases were not affected in growth or cell division (25, 33).

Presumably because PG monomers are usually efficiently recycled, only a few bacteria are known to release PG monomers during growth. These include Neisseria gonorrhoeae (62) and Bordetella pertussis (57), which cause gonorrhea and whooping cough, respectively. These pathogens each release N-acetylglucosaminyl-1,6-anhydro-N-acetylmuramylalanyl-γ-glutamyldiaminopimelylalanine (referred to herein as “PG monomer”), which triggers the death of ciliated host cells (12, 38, 42), but the basis for their shedding of PG monomers differs. B. pertussis releases of PG monomer, or “tracheal cytotoxin” (TCT), apparently is due to disruption of ampG expression by the insertion of an IS481 element 90 bp upstream of this gene (36), and artificially expressing ampG in B. pertussis.
decreased PG monomer release (36, 43). However, in *N. gonorrhoeae* the activities of the lytic transglycosylases LtgA (9) and LtgD (10) appear to be responsible for the release of PG monomer or “PG cytokinesis” (PGCT). An *ampG* mutant in *N. gonorrhoeae* showed a seventeenfold increase in PG monomer release, suggesting a functional AmpG and PG recycling pathway (18). Unfortunately, humans are the host for *N. gonorrhoeae* and *B. pertussis*, hindering examination of the effects of PG monomer during natural infections.

Recently, the bioluminescent marine bacterium *Vibrio fischeri* was found to release the same PG monomer as that released by *N. gonorrhoeae* and *B. pertussis* (31). *V. fischeri* is a light-organ symbiont of the Hawaiian bobtail squid, *Euprymna scolopes*, and this host-bacterium association can be reconstituted in the laboratory. *E. scolopes* hatchlings contain ciliated appendages that increase water flow across the light organ and help facilitate infection (40, 41, 48). During initial colonization, *V. fischeri* triggers regression of these ciliated fields (15, 47), and this can be mimicked by adding PG monomers to the seawater (31). In this study, we constructed and analyzed mutants that increase water flow across the light organ and *V. fischeri* DTCT) was also phenylisothiocyanate derivatized and run to compute picograms based on predicted product size. The annealing temperature for each primer was usually determined by subtracting 5°C from the lower calculated primer melting temperature. PCR was performed using an iCycler (Bio-Rad Laboratories, Hercules, CA). DNA sequencing was conducted on an ABI automated DNA sequencing analyzer at the University of Michigan DNA Sequencing Core, and sequences were analyzed using Sequencher 4.6 (Gene Codes, Ann Arbor, MI). Oligonucleotides (Table 1) were obtained from Integrated DNA Technologies (Corvalle, IA).

**Mutation construction.** Descriptions of select plasmids and the primers used in their construction are provided in Table 1. Construction of mutant strains and alleles is outlined in Table 1, and a summary follows. To generate plasmid insertion mutants, an internal fragment of each targeted gene was PCR amplified and either cloned directly into pEVSI22 (a vector that does not replicate in *V. fischeri*) or cloned into pCR-BluntII TOPO and subsequently subcloned into pEVSI12. The resulting plasmids were mobilized into *V. fischeri*, and plating on LBS-Em selected for transconjugants that had undergone homologous recombination between the genome and the internal gene fragment, enabling us to isolate vector integration mutants. In this way, plasmids pDMA48, pDMA49, pDMA90, pDMA91, pDMA108, and pDMA109 were used to generate mutants in *ampG*, *ltgA*, *ltgB*, *ltgC*, *ltgD*, and *ltgE*, respectively. In-frame *ampG*, *ltgA*, and *ltgD* deletion alleles were constructed in plasmids pDMA110, pDMA187, and pDMA199, respectively, such that the region between the start and stop codon of each target gene was replaced by a 6-bp restriction enzyme recognition site. These in-frame deletion mutations were placed in ES114 or in mutant backgrounds by allelic exchange. All mutants were confirmed by PCR. Constructs were generated in *E. coli* and transferred to *V. fischeri* by triparental mating using *E. coli* CC118 Δpir EVSI104 as a conjugative helper plasmid (65).

**Bioluminescent analyses.** Protein sequence comparisons to GenBank entries were generated using BLAST-P (2) and the BLOSUM62 scoring matrix (26). Genomes with similar regions surrounding the lytic transglycosylase open reading frames (ORFs) were found using the SEED pinned region search (54). To assess genetic context within the *Vibriocinaceae* family, we compared local gene arrangement in *V. fischeri* ES114 (59) to that in the genomes of *Vibrio alginolyticus* 12G01, *Vibrio anguillarum* S14, *Vibrio campbellii* AND4, *Vibrio cholerae* O1 biovar eltor strain N16961 (23), *Vibrio harveyi* ATCC BAA-1116, *Vibrio para-haemolyticus* RIMD 2210633 (37), *Vibrio vulnificus* CMCP6 (8), and *Photobacterium profundum* SS9 (69). Sequence alignments were performed with MEGA 4.0 using the Clustal method (66). The similarity and identity between homologs were determined with MatGAT using the default settings (7).

**PG monomer detection and quantification.** Culture supernatants were subjected to solid-phase extraction and reversed-phase high-pressure liquid chromatography (HPLC) (as described previously (12, 31). Briefly, culture supernatants were adjusted to pH 5.5, centrifuged (4°C, 10,000 x g, 10 min), and reloaded onto a 30-μm aspartic acid analyte cartridge (A&A). The elution buffer (1% TFA in water) was passed through the cartridge, and the eluate was concentrated and derivatized with 1% phenylisothiocyanate (PITC) and analyzed on a HPLC. A PG monomer standard (3.9 nM) was added to each sample before analysis to correct for any potential for loss during sample preparation or analysis (21). Each sample was separated on a 30-μm aspartic acid analyte cartridge (A&A) and analyzed on an Agilent 1100 HPLC system. The mobile phase consisted of an aqueous gradient, which included 0.1% trifluoroacetic acid and 3% acetonitrile. The mobile phase was delivered at a rate of 1 ml/min. The gradient (ratio of buffer A to buffer B) was as follows: 0 min, 100% buffer A; 1 min, 100% buffer B; 3 min, 100% buffer A; 4 min, 5% buffer B; 5 min, 92% buffer A; 8 min, 92% buffer A; 10 min, 100% buffer B; 13 min, 92% buffer A; 15 min, 0% buffer B; 35 min, 0% buffer B; and 36 min, 92% buffer A. The gradient was achieved by mixing buffer A and buffer B at the following rates: 0 to 100% at a rate of 1 ml/min. The gradient was completed in an injection volume of 2.0 μl. The eluate was monitored at 210 nm (OD210), with PG monomer being below the limit of detection (−1.5 nM OD210) in three of these experiments.
TABLE 1. Bacterial strains, plasmids, and oligonucleotides

<table>
<thead>
<tr>
<th>Strain, plasmid, or oligonucleotide</th>
<th>Relevant characteristics&lt;sup&gt;ab&lt;/sup&gt;</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacterial strains</strong>&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC1104pir</td>
<td>Δ(ara-leu) araD Δlac74 galE galK phoA20 thi-1 rpsE rpsB argEAm recA xpr</td>
<td></td>
</tr>
<tr>
<td>DH5a</td>
<td>F&lt;sup&gt;−&lt;/sup&gt; φ80lacZΔZamF1(argF)U169 deor SupF44 hsdR17 recA1 endA1 araG96 thi-1 recA1</td>
<td>22</td>
</tr>
<tr>
<td>DH5a pir</td>
<td>F&lt;sup&gt;−&lt;/sup&gt; Δ::gff309 pir C. Gross</td>
<td>16</td>
</tr>
<tr>
<td>MG1655</td>
<td>F&lt;sup&gt;−&lt;/sup&gt; Δ::gff309 pir C. Gross</td>
<td></td>
</tr>
</tbody>
</table>

**TOP10**

F<sup>−</sup> <sup>+</sup> mcrA Δ(ori4-110-mdmR5-mcrBC) φ80lacZΔlac15 ΔlacX74 recA1 araΔ139 (ara-leu)7697 galU galK rpsL (Str<sup>+</sup>)

endA1 nupG

**V. fischeri**

AKD200 ES114 mini-Tn7 insertion; Km<sup>+</sup> A. Dunn

DM131 ES114 flaL::aph; Km<sup>+</sup> D. Millikan

DMA350 ES114 ampC::pDMA48; Em<sup>+</sup> This study

DMA352 ES114 ΔampG (allele exchanged from pDMA110 into ES114) This study

DMA354 D3131 ΔampG (allele exchanged from pDMA110 into ES114); Km<sup>+</sup> This study

DMA360 ES114 lgtE::pDMA89; Em<sup>+</sup> This study

DMA361 ES114 lgtD::pDMA90; Em<sup>+</sup> This study

DMA362 ES114 lgtD::pDMA91; Em<sup>+</sup> This study

DMA363 ES114 ΔlgtD (allele exchanged from pDMA187 into ES114) This study

DMA368 ES114 ΔlgtD lgtE::pDMA90; Em<sup>+</sup> This study

DMA369 ES114 ΔlgtD lgtE::pDMA91; Em<sup>+</sup> This study

DMA380 ES114 ΔlgtE; Em<sup>+</sup> This study

DMA385 ES114 lysM::pDMA108; Em<sup>+</sup> This study

DMA386 ES114 ΔlgtE lgtD::pDMA90; Em<sup>+</sup> This study

DMA387 ES114 ΔlgtD lgtD::pDMA90; Em<sup>+</sup> This study

DMA388 ES114 ΔlgtD lgtD::pDMA90; Em<sup>+</sup> This study

ES114 Wild-type isolate from E. scolopes 3

**Plasmids**

pCR-BluntII TOPO

TOPO PCR cloning vector; Km<sup>+</sup> Invitrogen

pDMA46 Internal ampG fragment (PCR product; primers dma30 and dma31, ES114 template) in pCR-BluntII TOPO; Km<sup>+</sup> This study

pDMA48 pDMA46 BamHI fragment in BamHI-digested pEV5122; internal ampG fragment oriV<sub>RSK</sub>, oriT<sub>RPS</sub> Em<sup>+</sup> This study

pDMA74 Internal lgtE fragment (PCR product; primers dma58 and dma59, ES114 template) in pCR-BluntII TOPO; Km<sup>+</sup> This study

pDMA75 Internal lgtY fragment (PCR product; primers dma60 and dma61, ES114 template) in pCR-BluntII TOPO; Km<sup>+</sup> This study

pDMA76 Internal lgtD fragment (PCR product; primers dma62 and dma63, ES114 template) in pCR-BluntII TOPO; Km<sup>+</sup> This study

pDMA77 ampG complementing fragment (PCR product; primers dma52 and dma53, ES114 template) in pCR-BluntII TOPO; Km<sup>+</sup> This study

pDMA89 pDMA74 BglII fragment in BamHI-digested pEV5122; internal lgtE fragment; oriV<sub>RSK</sub>, oriT<sub>RPS</sub> Em<sup>+</sup> This study

pDMA90 pDMA75 BglII fragment in BamHI-digested pEV5122; internal lgtY fragment; oriV<sub>RSK</sub>, oriT<sub>RPS</sub> Em<sup>+</sup> This study

pDMA91 pDMA76 BglII fragment in BamHI-digested pEV5122; internal lgtD fragment; oriV<sub>RSK</sub>, oriT<sub>RPS</sub> Em<sup>+</sup> This study

pDMA96 ~1.5-kb fragment downstream of ampG stop (PCR product; primers 55 and dma57, ES114 template) in Smal-digested pEV5129; oriC<sub>RSK</sub>, oriT<sub>RPS</sub> Cm<sup>+</sup> This study

pDMA100 ~1.5-kb fragment upstream of ampG start (PCR product; primers dma54 and dma56, ES114 template) in HpaI-digested pJB103; oriV<sub>RSK</sub>, Km<sup>+</sup> This study

pDMA104 Internal lgtF fragment (PCR product; primers dma76 and dma77, ES114 template) in pCR-BluntII TOPO; Km<sup>+</sup> This study

pDMA105 pDMA96 Smal digested fused to Smal-digested pDMA100; oriV<sub>RSK</sub>, oriT<sub>RPS</sub> Km<sup>+</sup> Cm<sup>+</sup> This study

pDMA106 pDMA77 SpeI-Xbal fragment in SpeI-Xbal-digested pEV5129; oriC<sub>RSK</sub>, oriT<sub>RPS</sub> Cm<sup>+</sup> This study

pDMA108 pDMA104 BglII fragment in BamHI-digested pEV5122; internal lgtM fragment; oriV<sub>RSK</sub>, oriT<sub>RPS</sub> Em<sup>+</sup> This study

pDMA109 Internal lgtD fragment (PCR product; primers dma78 and dma79) BamHI-digested in BamHI-digested pEV5122; oriV<sub>RSK</sub>, oriT<sub>RPS</sub> Em<sup>+</sup> This study

pDMA110 pDMA105 AvrII-SpeI digested and self-ligated; ΔampG allele; oriC<sub>RSK</sub>, oriT<sub>RPS</sub> Cm<sup>+</sup> This study

pDMA115 pDMA106 SpeI-XbaI fragment in AvrII-digested pPV5120; V. fischeri ampG in shuttle vector pPV5120; oriV<sub>RSK</sub>, oriT<sub>RPS</sub> ori<sub>RSK</sub> Cm<sup>+</sup> This study

pDMA126 ~1.5-kb fragment upstream of lgt4 start (PCR product; primers dma95 and dma99, ES114 template) in pCR-BluntII TOPO; Km<sup>+</sup> This study

pDMA177 lgt4 complementing fragment (primers dma97 and dma98, ES114 template) in pCR-BluntII TOPO; Km<sup>+</sup> This study

pDMA185 ~1.5-kb fragment downstream of lgt4 stop (PCR product; primers dma96 and dma100, ES114 template) in pCR-BluntII TOPO; Km<sup>+</sup> This study

pDMA186 pDMA182 Xhol digested and self-ligated; oriV<sub>RSK</sub>, oriT<sub>RPS</sub> Cm<sup>+</sup> This study

pDMA187 Smal fragment in Smal-digested pDMA186; Δg4 allele; oriV<sub>RSK</sub>, oriC<sub>RSK</sub>, oriT<sub>RPS</sub> Cm<sup>+</sup> This study

pDMA191 ~1.5-kb fragment downstream of lgtD stop (PCR product; primers dma102 and dna106, ES114 template) in pCR-BluntII TOPO; Km<sup>+</sup> This study

pDMA196 pDMA177 SpeI-XbaI fragment in SpeI-XbaI-digested pPV5107; lgt4 in shuttle vector pPV5107; oriV<sub>RSK</sub>, oriT<sub>RPS</sub> ori<sub>RSK</sub> Cm<sup>+</sup> This study

pDMA197 ~1.5-kb fragment upstream of lgtD start (PCR product; primers dma105 and dma115, ES114 template) in pCR-BluntII TOPO; Km<sup>+</sup> This study

pDMA198 pDMA191 KpnI-Xbal fragment in KpnI-Xbal-digested pEV5129; oriC<sub>RSK</sub>, oriT<sub>RPS</sub> Cm<sup>+</sup> This study

pDMA199 pDMA198 Smal digested fused to Smal-digested pDMA197; Δg4 allele; oriC<sub>RSK</sub>, oriV<sub>RSK</sub>, oriT<sub>RPS</sub> Em<sup>+</sup> This study

pEV97 oriV<sub>RSK</sub>, oriT<sub>RPS</sub> Km<sup>+</sup> 65

pEV97 oriV<sub>RSK</sub>, oriT<sub>RPS</sub> Km<sup>+</sup> 65

pEV100 oriV<sub>RSK</sub>, oriT<sub>RPS</sub> Km<sup>+</sup> 17

pEV109 oriV<sub>RSK</sub>, oriT<sub>RPS</sub> Km<sup>+</sup> 17

pPV5102 oriV<sub>RSK</sub>, oriT<sub>RPS</sub> ori<sub>RSK</sub> Km<sup>+</sup> gfP This study

pPV5104 oriV<sub>RSK</sub>, oriT<sub>RPS</sub> ori<sub>RSK</sub> Km<sup>+</sup> gfP This study

pPV5107 oriV<sub>RSK</sub>, oriT<sub>RPS</sub> ori<sub>RSK</sub> Km<sup>+</sup> gfP This study

Continued on following page
Luminescence and motility assays. Motility and luminescence were assessed as described previously (1). Luminescence of cells cultured in SWTO (4) at 24°C was determined using a TD 20/20 luminometer (Turner Designs, Sunnyvale, CA). For motility assays, cultures were grown to mid-log phase (OD$_{595}$ of 0.5) at 24°C, 5°/H9262 and cultures for inoculation were grown as 1:1 mix of the symbiont relative to the wild type, animals were exposed to a 1:1 mix of the wild-type and mutant strains as described above. At the specified times after inoculation, infected and aposymbiotic animals were stained with both Cell Tracker orange and Tubulin Tracker green (Invitrogen, Carlsbad, CA) for approximately 1 h. After staining, animals were anesthetized by adding an equal volume of 0.37 M MgCl$_2$ and dissected to expose the light organ on the ventral side. Regression of the ciliated epithelial appendages was determined by epifluorescence microscopy with a Nikon (Melville, NY) Eclipse E600 microscope using a 51000 V2 fluorescence isoithiocyanate-tetramethyl rhodamine isocyanate dual-label filter set (Chroma Technology Corp., Rockingham, VT) and/or a Nikon Coolpix 5000 camera to obtain the epifluorescence images shown of animals stained with Cell Tracker. Regression stage (stages 0 to 4) was scored blindly, as set forth by Doino and McFall-Ngai (15). The E. scolopes light organ is bilobed, and each of the two lobes was scored separately, although we never observed an animal with lobes at different stages of regression. Images taken by scanning electron microscopy (SEM) are provided to help illustrate the appearance of regression stages, but animals were not scored for regression by SEM in this study.

RESULTS Bioinformatic analysis of PG synthesis and recycling in V. fischeri. Before the 921-Da PG monomer known as TCT (13, 68), PG monomer apparently is not generated during de novo PG synthesis but rather is produced during PG remodeling and consumed by recycling pathways (55). To examine whether de novo PG synthesis or PG recycling might be substantively different in V. fischeri, we examined its genome and found homologs of the E. coli PG synthesis and recycling pathways (see Table S1 in the supplemental material). Only minor deviations from the E. coli PG processing systems were evident. V. fischeri possesses only one homolog (AmIB) of the four amidases that may process PG monomer in the periplasm of E. coli (24, 68),
but it seems reasonable that one such amidase might be sufficient. *V. fischeri* also lacks a clear homolog of LdcA, an L,D-carboxypeptidase found in the *E. coli* PG recycling pathway (67); however, it does appear to encode distinct peptideases not found in *E. coli*, including a putative PG-targeting peptidease (encoded by ORF VF0720). Moreover, all of the functions downstream of LdcA in the recycling pathway appear intact. Based on these results, we speculated that *V. fischeri*’s net release of PG monomer might parallel either the poor *ampG* expression of *B. pertussis* or the high activity of lytic transglycosylases of *N. gonorrhoeae*, and we investigated these possibilities further.

**Analysis of AmpG in *V. fischeri***. The single AmpG homolog found in BLAST searches of the *V. fischeri* genome is encoded by ORF VF0720, which shares 26% identity and 49% similarity with *E. coli* AmpG. The *V. fischeri* AmpG amino acid sequence is shorter than that of *E. coli* at the C terminus by 73 amino acids; however, *N. gonorrhoeae* AmpG is similarly shorter than *E. coli* AmpG by 77 amino acids, yet it has demonstrated functionality (18).

**Mutation of ampG increases the net release of PG monomers**. To test the functionality of *V. fischeri* AmpG in PG monomer recycling, we used an HPLC-based method for PG monomer detection (12, 31) to analyze the amount of PG monomer accumulated extracellularly during log-phase growth of DMA350 (*ampG::pDMA48*) relative to that of the wild type. We saw an increase in net PG monomer release from DMA350 (*ampG::pDMA48*) of approximately 39-fold (data not shown). Next, we generated an in-frame deletion mutant of *ampG* and found that DMA352 (*ΔampG*) had more than a 100-fold increase in net PG monomer release (Fig. 1). This phenotype could be complemented by the introduction of *ampG* on a stable plasmid (pDMA115), decreasing the extracellular levels of PG monomer accumulation back to wild-type levels (Fig. 1). We occasionally saw a decrease in the amount of net PG monomer release when *ampG* was added in multi-copy in trans to ES114; however, this result was not consistent from experiment to experiment, and this apparent slight effect is not statistically significant (*P* > 0.05; Fig. 1). These data suggest that *V. fischeri* AmpG is functional, and unlike the situation in *B. pertussis*, lack of AmpG is probably not responsible for the extracellular accumulation of PG monomers in cultures of *V. fischeri*.

**Analysis of lytic transglycosylases in *V. fischeri***. In *N. gonorrhoeae* the activity of lytic transglycosylases is responsible for the large amounts of PG monomer accumulated in culture supernatants. Therefore, we examined whether *V. fischeri* had homologs to the *N. gonorrhoeae* lytic transglycosylases, LtgA and LtgD, which are important for release of PG monomers during log-phase growth in *N. gonorrhoeae* (9, 10). Two homologs to the *N. gonorrhoeae* LtgA sequence were found in *V. fischeri*, and these are encoded by VF0558 and VF1329 (see Table S1 in the supplemental material). VF0558, now termed *ltgA*, is well conserved in sequence and genetic context within the family Vibrionaceae, and the predicted amino acid sequence for *V. fischeri* LtgA has 24% identity and 43% similarity to that for *N. gonorrhoeae* LtgA. VF0558 and *N. gonorrhoeae* LtgA were best matches in reciprocal genome searches, leading to the designation of VF0558 as *ltgA*. The other LtgA-like protein, encoded by VF1329, is now designated *ltgY* to distinguish it from *ltgA* (VF0558) while avoiding the names of genes such as *ltgB* or *ltgC* that encode more-dissimilar proteins in *N. gonorrhoeae*. The *ltgY* gene is related to *ltgA* but appeared absent from the other members of the Vibrionaceae family that we examined. The predicted amino acid sequence of *ltgY* shares 34% identity and 55% similarity with that of *V. fischeri* LtgA and 24% identity and 42% similarity with that of *N. gonorrhoeae* LtgA. Only one homolog to *N. gonorrhoeae* LtgD was found in *V. fischeri*, and this is encoded by VF1702. A reciprocal search found that in the *N. gonorrhoeae* FA1090 genome LtgD is likewise the protein most similar to that encoded by VF1702. This *V. fischeri* gene, now termed *ltgD*, has a conserved upstream genetic context within the Vibrionaceae family, although the downstream genetic context is different. The predicted amino acid sequence of *V. fischeri* LtgD had 30% identity and 48% similarity to the *N. gonorrhoeae* LtgD sequence.

We also searched the ES114 genome for other genes potentially encoding lytic transglycosylases. Two ORFs, VF1939 and VF1247, were found to encode LysM domains that are important in general PG binding and have the potential to be PG hydrolases (6). These ORFs were named *ltgE* and *lysM*, respectively. Based on genome comparisons, VF1247 (*ltgE*) encodes a reciprocal best match to both the membrane-bound lytic murein transglycosylase MltD in *E. coli* (see Table S1 in the supplemental material) and LtgE in *N. gonorrhoeae*. We have designated VF1247 as *ltgE* to be consistent with the nomenclature in *N. gonorrhoeae* (10).

**Mutations in lytic transglycosylase genes result in reduced net release of PG monomers**. To determine if any of these putative lytic transglycosylases were responsible for the release of PG monomers by *V. fischeri*, we generated plasmid integration mutants yielding DMA360 (*ltgA::pDMA89*), DMA361 (*ltgY::pDMA90*), DMA362 (*ltgD::pDMA91*), DMA380 (*ltgE::
Mutations in V. fischeri lytic transglycosylases result in a decrease in net PG monomer release in log-phase cultures. Values are the averages of three different experiments ± standard errors. The dotted line represents ES114 PG monomer release, defined as 100%.

(A) Plasmid-insertion mutants with disruptions in \( \textit{ltgA} \), \( \textit{ltgY} \), \( \textit{ltgD} \), or \( \textit{ltgE} \). The wild type averaged 28 nM per OD\(_{595}\). (B) Comparison of mutants with combinations of in-frame deletion mutations \( \Delta \textit{ltgA} \) and \( \Delta \textit{ltgD} \) and a plasmid-insertion mutation in \( \textit{ltgY} \). The wild type for this set of experiments averaged 14 nM per OD\(_{595}\).

In \( \textit{N. gonorrhoeae} \) multiple mutations in the lytic transglycosylase genes are necessary to drastically reduce the accumulation of PG monomer in cultures. To determine whether this might be the case in \( \textit{V. fischeri} \), we constructed in-frame deletions of \( \textit{ltgA} \) and \( \textit{ltgD} \), resulting in DMA363 (\( \Delta \textit{ltgA} \)) and DMA368 (\( \Delta \textit{ltgD} \)), respectively. These alleles were combined with the \( \textit{ltgY} \::\text{pDMA90} \) allele construct both in pairwise combinations and to generate a triple mutant. The \( \Delta \textit{ltgA} \) allele combined with other mutations resulted in significantly less extracellular accumulation of PG monomer. This was true for DMA369 (\( \Delta \textit{ltgA} \Delta \textit{ltgD} \)), DMA386 (\( \Delta \textit{ltgA} \Delta \textit{ltgY} \::\text{pDMA90} \)), and DMA388 (\( \Delta \textit{ltgA} \Delta \textit{ltgD} \Delta \textit{ltgY} \::\text{pDMA90} \)), the last of which has very low (\( 2 \pm 1 \) nM per OD\(_{595}\)) levels of PG monomer in culture supernatants (Fig. 2B).

It is not clear why the \( \Delta \textit{ltgA} \) mutation appears to have a greater effect on PG monomer release (Fig. 2A) than does the \( \Delta \textit{ltgA} \) allele (Fig. 2B). We cannot rule out the possibility that allelic differences affect PG monomer release—for example, if a truncated \( \textit{ltgA} \) in the \( \textit{ltgA} \::\text{pDMA89} \) mutant interfered with other lytic transglycosylases; however, this apparent difference between mutants may simply reflect variability in this assay, as the \( \textit{ltgA} \::\text{pDMA89} \) and \( \Delta \textit{ltgA} \) mutants were not examined together. In this regard, it should be noted that the amount of PG monomer measured in supernatants from the wild type was smaller for the experiment in Fig. 2B than it was for the experiment in Fig. 2A (see figure legend), and the unusually low value for the wild type in Fig. 2B alone could explain why PG monomer released by the mutants expressed as a percentage of the wild-type value would appear higher in this data set.

**Growth, motility, and luminescence of ampG and lytic transglycosylase mutants.** Mutations affecting recycling and remodeling of PG could affect cell division, growth, motility, and even metabolism. However, all mutants had wild-type-like growth, cell morphology, and bioluminescence (data not shown), indicating that these are not generally attenuated strains. Mutants also had at least 80% to 99% motility relative to that of the wild type (data not shown), and a previous study found that these swimming rates are sufficient to confer full symbiotic competence (1).

**Symbiotic competence of DMA352 (\( \textit{ampG} \)) and DMA388 (\( \Delta \textit{ampG} \Delta \textit{ltgD} \Delta \textit{ltgY} \::\text{pDMA90} \)).** One of our goals was to examine the effect of altered extracellular PG monomer accumulation on symbiotic colonization. We therefore examined the symbiotic competence of the mutants with the greatest deviations in net PG monomer release; DMA352 (\( \Delta \textit{ampG} \)) and DMA388 (\( \Delta \textit{ampG} \Delta \textit{ltgD} \Delta \textit{ltgY} \::\text{pDMA90} \)). In single-strain infections of squid, neither DMA352 nor DMA388 showed a difference from the wild type (data not shown). Competition experiments have been used previously as a measure of relative symbiotic proficiency, revealing colonization defects not apparent in single-strain inoculations (4, 29, 32, 34, 35, 46, 53, 64, 70, 71, 74, 75). However, there was no apparent competitive defect 48 h or 96 h postinoculation when either DMA352 (\( \Delta \textit{ampG} \)) or DMA388 (\( \Delta \textit{ampG} \Delta \textit{ltgD} \Delta \textit{ltgY} \::\text{pDMA90} \)) was coinoculated along with the wild type (data not shown).

**Mutant effects on host light-organ morphogenesis.** Because PG monomers act as a morphogen triggering regression of the ciliated appendages on the light organ (31), we investigated whether mutations affecting the net amount of PG monomer released would alter this morphogenesis. Animals inoculated with DMA352 (\( \Delta \textit{ampG} \)) and examined at 24-h intervals up to 120 h showed ciliated appendage regression similar to that in animals infected with the wild type. Thus, the 100-fold increase in net PG monomer release by the \( \textit{ampG} \) mutant in...
culture did not appear to correlate with more rapid regression of the ciliated appendages.

We next examined whether the ampG mutation could trigger morphogenesis in a strain that could not colonize the light-organ crypts. Nonmotile V. fischeri strains do not enter the light-organ crypts and do not induce regression (15), although they are able to induce mucus production from the ciliated fields and can form aggregates outside the pores of the light organ (53). We therefore tested whether a nonmotile strain generating large amounts of extracellular PG monomer might be able to induce regression without colonizing the light-organ crypt, by constructing a flaJ ampG double mutant. We then compared morphogenesis in animals inoculated with the wild type, DM131 (flaJ::aph), or DMA354 (ΔampG flaJ::aph). To ensure that the nonmotile flaJ mutants did not infect the light-organ crypts, each of the three strains used was labeled with the stable gfp-expressing plasmid pSV102 (17), and epifluorescence microscopy confirmed that only wild-type cells could be visualized colonizing the crypts.

At 72 and 96 h postinoculation, wild-type-infected animals all displayed regression of the ciliated epithelial appendages (Fig. 3). In contrast, animals infected with DM131 (flaJ::aph) displayed essentially no regression (a few animals were scored at stage 1 regression), consistent with previous reports comparing the wild type to nonmotile mutants (21, 45). However, more than half of the animals inoculated with DMA354 (ΔampG flaJ::aph) showed regression, with some animals progressing as far as stage 3 (Fig. 3), even though the flaJ mutation blocked invasion of the light-organ crypts. We considered the possibility that PG monomer already present in the inoculum might be inducing regression, as Koropatnick et al. observed with as little as 10 nM purified PG monomer (31); however, assuming that net PG monomer release was similar under the growth conditions used to prepare inocula to the net PG monomer release in minimal media, then carryover of PG monomer from the inoculum should have resulted in animals being exposed to only ~10 pM PG monomer in these experiments. Moreover, similar results were obtained when DMA354 (ΔampG flaJ::aph) cells were washed in Instant Ocean before hatchlings were exposed to them. This result suggests that the regression induced by DMA354 (ΔampG flaJ::aph) is due to bacteria aggregating and shedding high levels of PG monomer in situ, on the light organ.

Our next goal was to see if the small amount of net PG monomer release by the lytic transglycosylase mutant DMA388 (ΔltgA ΔltgD ΔgycY::pDMA90) would reduce the stimulation of morphogenesis. It did appear that there was less regression of the ciliated appendages in animals infected with DMA388 (ΔltgA ΔltgD ΔgycY::pDMA90) than in animals infected with the wild type; however, using the semiquantitative scoring of regression stages 0 to 4 defined previously (15), we did not see a consistent and statistically significant difference in these treatments (data not shown). We also observed what appeared to be more dense and active fields of cilia on the appendages of DMA388-
infected animals, but again we were unable to quantify and adequately test this supposition. We therefore sought another way to measure the status of the ciliated appendages. We surmised that, if some animals retained their infection-promoting ciliated appendages longer, these animals might be more susceptible to a secondary infection with another bacterial strain presented well after initial infection. To address this experimentally, animals were inoculated following hatching with either the wild-type strain or DMA388 (ΔltgA ΔltgD ΔltgY:pDMA90), and at 72 h after inoculation with the primary strain, animals were exposed to a secondary inoculant strain, AKD200 (mini-Tn7 Cm'). At 120 h post-primary inoculation (i.e., 48 h post-secondary inoculation), animals were homogenized and plated on LBS to assess total colonization and plated on LBS-Cm to enumerate bacteria from the secondary infection. DMA388-infected animals had 10-fold-more bacteria from the secondary AKD200 infection than did wild-type-infected animals (Fig. 4A and B) and were complemented by the addition of ΔltgA on a stable multicopy plasmid (Fig. 4C and D).

Thus, DMA388-infected animals are more prone to secondary infection, consistent with our perception that the infection-promoting ciliated appendages were more intact in these animals.

**DISCUSSION**

A specific 921-Da component of gram-negative PG referred to here as PG monomer (and elsewhere as TCT or PGCT) is usually recycled by bacterial cells, but in instances where PG monomer is released in significant amounts, it elicits dramatic effects on host epithelial tissues. When released by the pathogen *B. pertussis* or *N. gonorrhoeae*, PG monomer causes severe cytopathology in ciliated human epithelial cells (12, 42). PG monomer is also released by the mutualistic symbiont *V. fischeri*, and in its natural host squid, purified PG monomer triggers regression of ciliated epithelial appendages of the symbiotic

![Graph](image)

**FIG. 4.** Animals infected with DMA388 (ΔltgA ΔltgD ΔltgY:pDMA90) are more susceptible to a secondary infection. Hatchlings were inoculated with either ES114, DMA388 (ΔltgA ΔltgD ΔltgY:pDMA90), DMA388 pSV107, or DMA388 pDMA196 (ΔltgA). After 72 h, animals were inoculated with the secondary strain AKD200 (mini-Tn7 Cm'). At 120 h, animals were homogenized and plated to determine both total CFU and Cm' CFU corresponding to the secondary colonizers. Values in panels A and B are the combined averages of six independent experiments, all of which yielded similar results (total n = 25 for ES114 and n = 26 for DMA388). Values in panels C and D are the combined averages of two independent experiments, which yielded similar results (total n = 9 for ES114, n = 10 for DMA388 pSV107, and n = 10 for DMA388 pDMA196). (A and C) Percentages of total infection comprised by the secondary colonist, AKD200. Each circle represents a single squid. Open circles represent animals below the limit of detection for Cm' cells. Horizontal lines show the averages of all animals within each treatment (panel A, 2% in ES114-infected animals, and 24% in DMA388-infected animals; panel C, 0.3% in ES114-infected animals, 7.4% in DMA388 pSV107-infected animals, and 0.2% in DMA388 pDMA196-infected animals). (B and D) Averages of both total CFU (open bars) and CFU from secondary infection by AKD200 (shaded bars). Error bars are standard errors. Student t tests indicated that secondary infection by AKD200 in DMA388-infected animals was significantly greater (P < 0.01) than that in ES114-infected animals in panel B and that secondary infection by AKD200 in DMA388 pSV107-infected animals was significantly greater (P < 0.05) than that in ES114- or DMA388 pDMA196-infected animals in panel D.
light organ, reminiscent of the normal developmental program induced in this symbiosis (31). In this study we generated mutants of *V. fischeri* that displayed either increased or decreased extracellular accumulation of PG monomer, providing insight into the basis for PG monomer shedding in this bacterium and the role of PG monomers in this model mutualistic infection.

We first examined the genetic determinants of PG monomer release in *V. fischeri*. In contrast to *B. pertussis*, where extracellular PG monomer accumulation is thought to be a result of poor *ampG* expression (36), *V. fischeri AmpG* functioned well in reducing net PG monomer release, as illustrated by the 100-fold increase in PG accumulated in the medium of an *ampG* mutant and by the observation that overexpressing *ampG* from *V. fischeri* or *E. coli* did little to lessen net PG monomer release (Fig. 1 and data not shown, respectively). *V. fischeri* apparently also has a complete recycling pathway (see Table S1 in the supplemental material), suggesting that *V. fischeri* recycles PG monomers as other gram-negative bacteria do. Apparently, the basis for *V. fischeri*’s extracellular accumulation of PG monomers is more similar to that of *N. gonorrhoeae*, where the deletion of two lytic transglycosylases results in the reduced net release of PG monomers (9, 10). In *V. fischeri*, mutation of multiple lytic transglycosylases similar to those in *N. gonorrhoeae* also reduced net PG monomer release (Fig. 2B).

The generation of mutants with more or less extracellular accumulation of PG monomer than that of the wild type enabled us to evaluate the importance of this molecule in vivo during symbiotic infection. When squid were inoculated with DMA352 (*ΔampG*), which accumulates 100-fold more PG monomers in culture supernatants than does the wild type (Fig. 1), we did not see a faster progression of light-organ morphogenesis (data not shown). This is consistent with the idea that PG monomer elicits effects on the host as a triggering signal for this strain (*V. fischeri*). Apparently, the basis for *V. fischeri*’s extracellular accumulation of PG monomers is more similar to that of *N. gonorrhoeae* with a small amount of PG monomer released by this strain is sufficient to stimulate morphogenesis or that other PG fragments besides the 921-Da fragment could have an effect on regression. A reduced version of the PG monomer is also released by *V. fischeri* (data not shown), and this or other PG fragments might have important morphogenic activity.

Despite the morphogenesis induced by DMA388 (*ΔltgA ΔltgD ΔltgY::pDMA90*), we did perceive a subtle attenuation of this strain’s ability to stimulate regression of the host’s light-organ ciliated appendages. A more dramatic or at least more easily quantifiable effect was evident in the greater susceptibility of DMA388-infected animals to a secondary *V. fischeri* colonist after regression of the infection-promoting ciliated appendages had begun (Fig. 4). This suggests that even an apparently small difference in the progression of the ciliated appendages may have large functional significance with regard to preventing or allowing further infection by environmental bacteria. In the future it will be interesting to examine the effects of DMA388 on specific infection-promoting characteristics of the ciliated appendages, such as water movement and mucus secretion (41, 50–53).

The dramatic regression of the infection-promoting ciliated appendages on the *E. scolopes* light organ following successful infection with *V. fischeri* symbionts can be easily rationalized as an advantage for the host. This morphogenesis effectively deters further infection, and if these infection-promoting structures carry any risk of promoting detrimental pathogenic infections, then the risks associated with these structures may outweigh the advantages once mutualistic symbionts are obtained. Our results illustrate how *V. fischeri* may also have an evolutionary impetus for shedding PG monomers. By stimulating morphogenesis, PG monomers help *V. fischeri* to essentially close the door behind them after initial infection. A secondary infection with another strain would create competition within the light organ, potentially causing the initial colonizer to lose some of the fitness advantage of colonizing the light organ. Initiating the regression of the ciliated epithelial appendages therefore enhances the initial colonizer’s ability to maintain dominance in the light organ. Further use of secondary-infection challenge assays should be valuable in the future, because mutants that lack the ability to prevent secondary infections could reveal genes important for stimulating regression of the ciliated appendages that may not be found using other screens.

**ACKNOWLEDGMENTS**

We thank Joshua Troll, Melissa Altura, and Amy Schaeffer for helpful discussions; Joseph Dillard for sharing data on *ltgD* before publication; William Swinehart for technical assistance; and Debbie Millikan, Edward Ruby, and Anne Dunn for providing strains. This work was supported by the National Institutes of Health under grant AI50661 to M.J.M.-N. and by the National Science Foundation under grant Career MCB-0347317 to E.V.S.
REFERENCES


