Modulation of *Neisseria gonorrhoeae* susceptibility to vertebrate antibacterial peptides due to a member of the resistance/nodulation/division efflux pump family

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ABSTRACT  We have previously described the antibacterial capacity of protegrin-1 (PG-1), a cysteine-rich, cationic peptide from porcine leukocytes, against *Neisseria gonorrhoeae*. We now report genetic and biochemical evidence that gonococcal susceptibility to the lethal action of PG-1 and other structurally unrelated antibacterial peptides, including a peptide (LL-37) that is expressed constitutively by human granulocytes and tests and inducibly by keratinocytes, is modulated by an energy-dependent efflux system termed mtr. These results indicate that such efflux systems may enable mucosal pathogens like gonococci to resist endogenous antimicrobial peptides that are thought to act during infection.

Researchers have isolated antibacterial peptides from numerous vertebrate, invertebrate, and plant sources during the past decade (1). These peptides represent part of the first line of host defense against invading pathogens and, for certain hosts, may represent the primary mechanism of host resistance against infectious agents (2). Antibacterial peptides are present constitutively in certain phagocytic cells (3). Their synthesis by epithelial cells, which line certain mucosal surfaces, can be induced by infection, inflammation, or trauma (4, 5). The broad-spectrum antimicrobial action of many peptides and the emergence of pathogens that are resistant to many currently available antibiotics have stimulated the development of antibacterial peptides as therapeutic agents (6).

We recently described the *in vitro* antibacterial actions of protegrins, a family of antimicrobial peptides found in porcine leukocytes (7–9). Protegrins also display potent broad-spectrum action *in vivo* in experimental murine infections (10). Like the well characterized defensins, protegrins are cationic, cysteine-rich peptides that assume a β-structure in solution. However, protegrins are smaller than defensins (16–18 vs. 29–40 amino acids, respectively) and have two instead of three intramolecular disulfide bridges (see Fig. 1). The intramolecular disulfide bonds of protegrins appear to be crucial for their maximal bactericidal activity because a linear synthetic protegrin variant (PC-8) that lacked the four invariant cysteines found in native protegrins was at least 100-fold less active against *Neisseria gonorrhoeae* (11) than the parental peptide, protegrin-1 (PG-1). The detailed, systematic structure-function analysis of PG-1 conducted by Qu et al. (11) suggested that the amphipathic central β-sheet domain of PG-1 (12) is essential for its bactericidal action against *N. gonorrhoeae*. Although the structurally related human and rabbit defensins fail to exert activity against *N. gonorrhoeae*, the protegrins display antigenococcal action at low micromolar concentrations (9). Electron microscopic studies revealed numerous surface-associated lesions on PG-1-treated gonococci, suggesting that these peptides are membrane-acting compounds. Thus, it may be difficult for pathogens such as gonococci to develop specific mechanisms of protegrin resistance. However, nonspecific mechanisms of microbial resistance such as reduced permeability or energy-dependent efflux systems (13) that enhance bacterial resistance to multiple antimicrobial agents might decrease levels of microbial susceptibility to antibacterial peptides.

Gonococci possess an energy-dependent efflux pump termed *mtr* (multiple transferable resistance) that confers enhanced resistance to structurally diverse, antimicrobial hydrophobic agents (HAs) (14–18), including membrane-damaging compounds (e.g., bile salts and fatty acids) that bathe certain mucosal surfaces. The *mtr* efflux pump is encoded by a three-gene, single transcriptional unit (*mtrCDE*) (14). The gonococcal MtrC-MtrD-MtrE proteins are highly similar to efflux pump proteins possessed by *Escherichia coli* and *Pseudomonas aeruginosa* (19) and have been placed in the resistance/nodulation/division (RND) family of efflux pumps (20).

The known capacity of the *mtr* efflux system to enhance gonococcal resistance to HAs, including cationic compounds that exert their antimicrobial action at the cytoplasmic membrane, prompted us to evaluate whether this mechanism of resistance might decrease gonococcal susceptibility to antimicrobial peptides. We now report that loss of the *mtr* efflux pump results in increased susceptibility of gonococci to PG-1 and LL-37, a recently described (5) α-helical human antimicrobial peptide expressed constitutively by granulocytes and tests and inducibly by keratinocytes.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. Table 1 is a description of the genotype and source of the isogenic gonococcal strains used in this investigation. Strain FA19 is the wild-type parental strain that was used in the genetic construction of strains KH12, KH14, and RD1 (14, 15, 18). Strain BR54 is a transformant of the FA19 genetic lineage constructed previously (21) and is hypersusceptible to HAs such as erythromycin, Triton X-100, and crystal violet. Strain WV2 is a spontaneous erythromycin-resistant mutant of BR54 that is cross-resistant to additional HAs (22). Strain BR54 contains a 10-bp deletion at the 3′-end of the *mtrD* gene (GenBank accession no. AF037040) that causes a frame shift after codon

Abbreviations: CCCP, carbonyl cyanide-m-chlorophenylhydrazone; HA, hydrophobic agents; *mtr*, multiple transferable resistance; MGIC, minimal growth inhibitory concentration; PG-1, protegrin-1; RND, resistance/nodulation/division; cfu, colony-forming unit; PMF, proton motive force.

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912, resulting in truncation of MtrD (938 vs. 1056 amino acids); this deletion has no effect on the downstream expression of the mtrE gene. Strain WV2 contains an additional 2-bp deletion in its mtrD gene that restores the correct reading frame of mtrD but leaves an internal four amino acid deletion (residues 912–916) in MtrD. All gonococcal strains were routinely cultured in their non-piliated, opacity-negative state on GCB typing agar (Difco) containing defined supplements I and II (23) under 3.8% (vol/vol) CO₂ at 37°C as described previously (23). Growth from GCB agar plates was resuspended in GCB broth containing defined supplements and 0.043% (wt/vol) sodium bicarbonate and grown with shaking at 37°C to mid-logarithmic phase.

Antibacterial Peptides. Fig. 1 shows the primary structures of the antibacterial peptides used in this study. PG-1 and PC-8 were synthesized as described previously (9, 11). Defensins HNP 2 and NP 2 were purified from human and rabbit granulocytes, respectively (24, 25). Tachyplesin-1 (TP-1) was purchased from Bachem (King of Prussia, PA). LL-37 was synthesized on a 0.25-mmol scale with a Perkin–Elmer Applied Biosystems model 431A synthesizer using FastMoc chemistry (26). A prederivatized polyethylene glycol polystyrene resin [Fmoc-L-Ser(tBu)-PEG-PS; PerSeptive Biosystems, Framingham, MA] was used for synthesis and all residues were single coupled. The final product was homogeneous by analytical reverse phase (RP)-HPLC and capillary zone electrophoresis coupled. The final product was homogeneous by analytical reverse phase (RP)-HPLC and capillary zone electrophoresis coupled.

RESULTS AND DISCUSSION

Genetic Evidence that Antibacterial Peptide Susceptibility in Gonococci Is Linked to the mtrCDE-Encoded Efflux Pump. Using the microtiter well growth inhibition assay described above, we found (Table 1) that isogenic transformant strains bearing insertionally inactivated mtrC (strain KH12), mtrD (strain KH14), or mtrE (strain RD1) genes were significantly more susceptible to PG-1 (MGICs range of 0.2–0.75 µg/ml) than parental strain FA19 (MGIC of 4.37 µg/ml). The extent of enhanced PG-1 susceptibility of these strains was similar to that observed previously for other antimicrobial compounds that are removed from gonococci by the mtr efflux system (14–18).

TABLE 1. Susceptibility of isogenic strains to PG-1

<table>
<thead>
<tr>
<th>Strain*</th>
<th>Relevant genotype</th>
<th>PG-1 MGIC, µg/ml†</th>
</tr>
</thead>
<tbody>
<tr>
<td>FA19</td>
<td>Wild type</td>
<td>4.37 (±0.88)</td>
</tr>
<tr>
<td>KH12</td>
<td>mtrC::Km</td>
<td>0.75 (±0.28)</td>
</tr>
<tr>
<td>KH14</td>
<td>mtrD::Km</td>
<td>0.69 (±0.22)</td>
</tr>
<tr>
<td>RD1</td>
<td>mtrE::Km</td>
<td>0.2 (±0.11)</td>
</tr>
<tr>
<td>BR54</td>
<td>mtrD54</td>
<td>0.67 (±0.31)</td>
</tr>
<tr>
<td>WV2</td>
<td>As BR54 but mtrD54‡</td>
<td>7.14 (±2.67)</td>
</tr>
</tbody>
</table>

*The construction of transformant strains KH12, KH14, and RD1 has been described (14–18). Strain BR54 was kindly provided by P. F. Sparling (21). Strain WV2 is a spontaneous mutant of BR54 that displays enhanced HA-resistance (22).

†As determined by the growth inhibition assay using 0.2× GCB broth with 20 to 0.15 µg of PG-1 per ml. The results are mean values (±SD) from at least four independent determinations.

‡The construction of transformant strains KH12, KH14, and RD1 has been described (14–18). Strain BR54 was kindly provided by P. F. Sparling (21). Strain WV2 is a spontaneous mutant of BR54 that displays enhanced HA-resistance (22).

The PG-1 hypersusceptible property of strain KH14 was studied in detail and verified by a colony-reduction plating assay (Fig. 2) that compared its susceptibility to that of parental strain FA19 in the presence of increasing concentrations of PG-1 (Fig. 2A) or a fixed amount (2 µg/ml) of PG-1 (Fig. 2B). Because the kanamycin-resistance cassette within the mtrD gene, which encodes the membrane transporter ionization mass-spectrometry. Peptides were purified by RP-HPLC, lyophilized, and dissolved in 0.01% (vol/vol) glacial acetic acid. Dissolved peptides were stored at −20°C and were thawed immediately before use.

Antibacterial Assays. Mid-logarithmic phase cultures of gonococci were typically diluted 100-fold in normal, 0.2×, or 0.1× strength GCB broth, all of which lacked the glucose and iron supplements described above. Ninety microliters of the diluted cultures was added to sterile 96-well polypropylene microtiter wells that contained 10 µl of peptide solution. Different concentrations of peptide were achieved by 2-fold serial dilutions, using 0.01% (vol/vol) acetic acid, before addition of bacteria. Dilutions of bacteria from a control well that contained 10 µl of 0.01% acetic acid were plated at the beginning of the experiment and after a 45-min incubation period at 37°C under 3.8% (vol/vol) CO₂. At the conclusion of the incubation period, 2.5 µl of bacteria from each well was spotted onto GCB agar plates to determine the minimal growth inhibitory concentration (MGIC) of peptides or dilutions were plated to determine the absolute reduction in colony-forming units (cfu). In the latter case, the data were calculated as log₁₀ bacterial units (log₁₀ cfu/ml = 0 minus log₁₀ cfu/ml at 45 min). All assays were performed in triplicate. The data are reported as average values ± the standard deviation. Statistical significance was determined by Student’s t test.

The cfu reduction assay was modified to test the capacity of the proton conductor carbonyl cyanide-m-chlorophenylhydrazone (CCCP) to enhance bacterial susceptibility to antibacterial peptides; CCCP was purchased from Sigma. Diluted gonococci (strain FA19) in microtiter wells were exposed to CCCP (50 µM) for 20 min at 37°C, treated with glucose (20 mM) or buffer for 10 min, and then exposed to a sublethal concentration of the test peptide. After 30 min of additional incubation at 37°C, dilutions from each sample were plated onto GCB agar.

Accumulation of Iodinated PG-1 by Gonococci. Four hundred micrograms of PG-1 was iodinated to a specific activity of 9 × 10⁶ dpm/µg using Iodogen-coated tubes (Pierce) and 500 µCi (1 Ci = 37 GBq) of Na[¹²⁵I] (ICN), as described by the manufacturer. Unincorporated label was removed by extensive dialysis, which used tubing with a 500-Da exclusion limit (Spectrum, Houston), against 0.01% acetic acid at 4°C. (Iodination of PG-1 did not alter its antimicrobial action against the test gonococcal strains.) The accumulation of a sub-MGIC amount (0.25 µg/ml) of [¹²⁵I] PG-1 with gonococci in the presence or absence of CCCP (50 µM) and/or glucose (20 mM) was assessed as described previously (16).
As an additional test of the above hypothesis, we next examined levels of PG-1 susceptibility among isogenic transformant strains bearing mutations in the mtrR gene (14, 28), which encodes a transcriptional repressor [MtrR (17)] that regulates transcription of the mtrCDE operon (28). Enhanced resistance of gonococci to HAs can be due to mutations in the mtrR gene, either as a result of a single basepair deletion in its promoter (28) or a missense mutation that causes a radical amino acid change in the helix–turn–helix motif of MtrR that abrogates binding of it to the mtrCDE promoter (29). Thus, isogenic transformants of strain FA19 or clinical isolates bearing mutations in their mtrR genes (30) were more resistant to PG-1 than strain FA19 (data not shown), displaying MGICs to PG-1 of 10–20 μg/ml. Thus, enhanced expression of the mtr efflux pump can result in decreased gonococcal susceptibility to PG-1 in a fashion reminiscent of gonococcal susceptibility to HAs (14).

**Table 2.** Susceptibility of strains FA19 and KH14 to antibacterial peptides: MGICs of test strains under high and low salt

<table>
<thead>
<tr>
<th>Peptide</th>
<th>High salt*</th>
<th>Low salt†</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>FA19</td>
<td>KH14</td>
</tr>
<tr>
<td>PG-1</td>
<td>10</td>
<td>1.25</td>
</tr>
<tr>
<td>PC-8</td>
<td>&gt;100</td>
<td>100</td>
</tr>
<tr>
<td>LL-37</td>
<td>&gt;100</td>
<td>3.12</td>
</tr>
<tr>
<td>HNP 2</td>
<td>&gt;200</td>
<td>&gt;200</td>
</tr>
<tr>
<td>NP 2</td>
<td>&gt;200</td>
<td>&gt;200</td>
</tr>
<tr>
<td>TP-1</td>
<td>5</td>
<td>1.25</td>
</tr>
</tbody>
</table>

All values are expressed in μg/ml as determined by the growth inhibition assay described in text. All values are from a single assay that are representative of two-three independent determinations. The concentrations in μg/ml of each peptide tested were: PG-1, 20-0.15; PC-8, 100-1.56; LL-37, 100-0.375; HNP 2 and NP 2, 200-6.25; TP-1, 10-0.15.

*High salt = 76.5 mM NaCl.
†Low salt = 7.65 mM NaCl in the incubation media.
Loss of the Proton Motive Force (PMF) Enhances Gonococcal Susceptibility to Antibacterial Peptides. The above results suggested a role for the mtr efflux system in determining levels of gonococcal susceptibility to certain antibiotic-like peptides. Because the action of this pump requires the PMF (16), we asked whether its poisoning by the proton conductor CCCP, which depolarizes the cytoplasmic membrane (13, 31), would enhance gonococcal susceptibility to PG-1. CCCP has been used extensively by others (13, 19, 31) in their studies dealing with the PMF-dependent efflux of antibiotics and other antibacterial substances by bacteria. Typically, addition of CCCP to strains possessing PMF-dependent efflux pumps enhance accumulation of antibacterial compounds that are normally removed by the respective efflux system, by 2- to 4-fold. The loss of efflux activity due to the depolarizing effect of CCCP can be reversed by an appropriate energy source, such as glucose. In this respect, we previously showed (16) that CCCP-mediated loss of efflux of [3H]-Triton X-100 due to the gonococcal mtr system could be reversed by the addition of glucose. Accordingly, we determined the susceptibility of strain FA19 to PG-1 in the presence or absence of CCCP and/or glucose. Using a sub-MGIC amount of PG-1 (1 μg/ml), we found that gonococci pre-exposed to CCCP for 20 min were significantly (P = 0.014) more susceptible to subsequent exposure to PG-1 (Fig. 3) than gonococci pretreated with CCCP. However, when gonococci were treated with CCCP and then with glucose, susceptibility to PG-1 was significantly reduced compared with those cultures treated with CCCP, but not glucose, before addition of PG-1 (P = 0.013). In fact, the glucose-energized cultures displayed a susceptibility to PG-1 not significantly different (P = 0.15) from those that were exposed only to PG-1 (Fig. 3).

We next examined the capacity of CCCP to enhance gonococcal susceptibility to LL-37, defensins NP 2 and HNP 2, and PC-8. Treatment of strain FA19 with CCCP did not enhance gonococcal susceptibility to 100 μg/ml of either defensin (data not shown). In contrast, CCCP treatment did enhance gonococcal susceptibility to 50 μg/ml of PC-8 by more than one order of magnitude. The results from two independent experiments showed (data not shown) that treatment of gonococci with CCCP before PC-8 addition resulted in 2.05 log10 reduction in viability as opposed to a 0.55 log10 reduction in the absence of CCCP. Addition of glucose to the CCCP-treated culture restored gonococcal resistance to PC-8 (0.95 log10 reduction in viability). Similar experiments with the antigono-
coccal LL-37 peptide showed that strain FA19 could be rendered more susceptible to LL-37 after treatment with CCCP. Thus, gonococci exposed to CCCP and then a normally sub-MGIC amount of LL-37 (0.5 μg/ml) were significantly (P = 0.002) more susceptible to this human peptide [log10 reduction in viability of 2.14 (±0.0085)] than in the absence of CCCP [log10 reduction = 0.243 (±0.016)]. The effect of CCCP on enhancing gonococcal susceptibility to LL-37 could be reversed by glucose addition [log10 reduction = 1.26 (±0.16)]; the difference between these samples was statistically significant (P = 0.0017).

Accumulation of PG-1 by Goncococi. The genetic and physiologic results described above implicated the energy-dependent mtr efflux pump in determining levels of gonococcal susceptibility to structurally diverse antibacterial peptides. Biochemical evidence in support of this hypothesis was obtained by determining the accumulation of a sub-MGIC amount (0.25 μg/ml) of [125I]PG-1 by gonococci. As is shown in Fig. 4A, treatment of strain FA19 with CCCP 15 min of incubation with PG-1 enhanced gonococcal accumulation of this peptide by 2-fold during a subsequent 20-min incubation period. This enhanced accumulation of PG-1 by strain FA19 could, however, be completely reversed by the addition of glucose 10 min after CCCP addition. Thus, loss of the gonococcal PMF due to the action of CCCP (16, 31) presumably inactivated the efflux action of the MtrC–MtrD–MtrE efflux pump, resulting in enhanced accumulation of PG-1. Once the cytoplasmic membrane was reenergized by glucose (16, 19, 31), the capacity of this efflux pump to export PG-1 was likely restored. It was verified that the MtrC–MtrD–MtrE efflux pump was indeed responsible for PG-1 export by comparing the accumulation of radiolabeled PG-1 (0.25 μg/ml) by isogenic strains FA19 and KH14, which revealed that strain KH14 accumulated at least 50% more PG-1 than parental strain FA19 over a 35-min incubation period (Fig. 4B). In triplicate, independent experiments (data not shown) that measured PG-1 accumulation by strains FA19 and KH14 after a 30-min exposure to 0.25 μg/ml of [125I]PG-1, we found that the difference between these isogenic strains [6.65 (±1.86) vs. 12.62 (±1.15) nmol, respectively] was indeed significant (P = 0.043).

The MtrC–MtrD–MtrE efflux pump can recognize a broad range of substrates that includes structurally diverse antibiotics, dyes, and detergents (14–18). Based on genetic and biochemical results obtained in this study, this list now includes certain antibacterial peptides that can assume β-sheet (PG-1) or α-helical (PC-8 and LL-37) structures. While there seems to be little in common among the diverse compounds recognized by this and other RND-type efflux pumps, they typically are hydrophobic compounds and some have a charged domain. Nikaido (19) virtually predicted that RND efflux pumps would modulate bacterial resistance to antibacterial peptides by indicating that these pumps are of importance in the bacterium’s attempt to remove toxic, foreign compounds that interact with the cytoplasmic membrane. Thus, because the protegrin class of antibacterial peptides (and probably the human LL-37 as well) are thought to reach the cytoplasmic membrane, a
should be expanded to encompass endogenous antibacterial salts that bathe certain mucosal surfaces (e.g., the rectum) allow it to resist the antibacterial effects of fatty acids and bile acids (17, 23, 30). The earlier hypothesis (33) that the MtrC–MtrD–MtrE pump can enhance gonococcal resistance to these membrane-active peptides is supported by the results presented herein, it is not surprising then that the MtrC–MtrD–MtrE pump can enhance gonococcal survival.

Unlike PG-1, LL-37 is a cysteine-free peptide that adopts an α-helical conformation. Given its tissue and fluid distribution, LL-37 is likely to be present at the genital mucosal surface during gonococcal infection. We propose that the antigonococcal action of the human LL-37 peptide is notable for its broad-spectrum activity against gonococcal isolates from multiple geographic locations, including those derived from humans. In this respect, the antigonococcal action of the human LL-37 peptide is noteworthy.

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N. gonorrhoeae is a strict human pathogen with a propensity to circumvent several host defensive systems (33). The earlier hypothesis (17, 23, 30) that the mtr efflux system evolved to allow it to resist the antibacterial effects of fatty acids and bile salts that bathe certain mucosal surfaces (e.g., the rectum) should be expanded to encompass endogenous antibacterial peptides, especially those—like LL-37—that gonococci might encounter on genitourinary mucosa.

![Graph A](image1.png)  
**Fig. 4.** (A) The accumulation of 0.25 μg/ml of [125I]PG-1 by strain FA19 in the absence of CCCP (○–○), after CCCP addition (△–△) or after glucose addition to the CCCP-treated culture (□–□); the time points of these additions are shown. (B) The accumulation of [125I]PG-1 (0.25 μg/ml) by isogenic strains FA19 (○–○) and KH14 (△–△). All values were calculated as nanomoles of PG-1 accumulated per 10^7 cfu.