

Chapter 17

Efflux Pumps in *Neisseria gonorrhoeae*: Contributions to Antimicrobial Resistance and Virulence

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Abstract This chapter deals with the structure-function relationships, genetics, gene regulation systems, and clinical/biologic significance of efflux pumps expressed by the sexually transmitted human pathogen *Neisseria gonorrhoeae*. The overarching theme emphasized herein is that bacterial efflux pumps contribute not only to the ability of *N. gonorrhoeae* to evade many antibiotics in current or past treatment regimens for gonorrhea but they also help this pathogen to evade antimicrobials that contribute to innate host defense during infection. Accordingly, gonococcal drug efflux pumps should be viewed not only in the context of their capacity to negatively impact antimicrobial therapies but in the larger picture as virulence factors that promote survival of *N. gonorrhoeae* during infection. Based on this

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hypothesis, we posit that strategies that cripple gonococcal efflux pump activities may prove useful in the design of new therapies, which is of special importance in this era when antibiotic-based treatment options for gonorrhea are dwindling due to mechanisms of bacterial resistance that include the action of drug efflux pumps.

Keywords *Neisseria gonorrhoeae* • Antimicrobial resistance • Efflux pumps • Regulation • Structure • Virulence • Vaccine • MtrCDE • MtrF • FarAB • MacAB • NorM • MtrA • MtrR • MpeR

17.1 Introduction

Neisseria gonorrhoeae is a strictly human, Gram-negative pathogen that is typically transmitted by sexual contact and causes the disease termed gonorrhea also known as “the clap,” which is a slang term thought to be derived from the name of the district Les Clapiers in Paris, France, that housed prostitutes in the late fourteenth century [1]. Gonorrhea typically presents as urethritis in men and cervicitis in women, but rectal and pharyngeal infections also occur in both sexes; the spectrum of clinical manifestations of gonorrhea has been reviewed recently [1]. If gonorrhea is left undetected, untreated, and inappropriately treated or fails to respond to conventional antibiotic treatment due to resistance (an important contemporary concern), the disease can ascend to the upper genital tract where it can cause severe reproductive complications (especially for women) such as endometritis, pelvic inflammatory disease, penile edema, and epididymitis. These complications can have a significant, devastating impact on general and reproductive health, including infertility, of infected individuals. Moreover, involuntary loss of life due to gonorrhea-associated ectopic pregnancy is not uncommon especially in the developing world. On rare occasions, gonococci can enter the bloodstream causing disseminated gonococcal infection and triggering damage to organs and joints distant from the site of infection. Conjunctivitis, more frequent in newborns (ophthalmia neonatorum) than adults, can result in blindness; transmission to the newborn can occur during vaginal delivery when the mother has cervical gonorrhea. It is also important to note that gonorrhea can increase transmission and host susceptibility to other sexually transmitted infections (STIs) including HIV, which emphasizes the importance of effective antimicrobial treatment regimens for gonorrhea. Thus, to prevent these medical issues and complications, it is imperative that effective and accessible antibiotic treatment and prevention regimens exist.

Historical writings suggest that gonorrhea has afflicted humans for thousands of years [1]. In this respect, a passage from the *Book of Leviticus* of the Old Testament warning women to avoid men with discharges and the writings of the second-century Greek physician Galen that described purulent exudates observed often in men with what is now considered as gonorrhea (“flow of seed”) suggest that this is an ancient disease; a more in-depth and recent historical review of gonorrhea can be

found in a recent review by Unemo and Shafer [1]. Presently, gonorrhea is without question a major public health problem as emphasized by the estimated 78 million infections worldwide per year and the emergence of strains resistant to first-line antibiotics [1–3]. The looming public health disaster of gonococcal resistance to current first-line antibiotics, combined with retained resistance to previous first-line antibiotics, and the lack of new, effective antibiotics that can serve as replacement therapies has been recently emphasized [3]. In this context, it is important to emphasize that in the absence of a vaccine, effective antibiotic therapy is crucial for reducing spread of gonorrhea in the community.

As a strict human pathogen, gonococci have over the millennia found ways to resist the multitude of innate and adaptive immune responses that occur during infection (summarized in [4]). Pathogens, like gonococci, encounter numerous host biocides (e.g., antimicrobial peptides, toxic-free fatty acids, bile salts, and progesterone), and their capacity to resist these antimicrobials likely promotes their survival and proliferation during infection. It is now evident that many of these antimicrobials are substrates for gonococcal multidrug transporters (see Sect. 17.7 below). Moreover, strong evidence exists that implicates gonococcal efflux pumps in exporting important antibiotics (e.g., β -lactams and macrolides) that are currently used in the clinic (see Sect. 17.6 [5]). In sum, studies on efflux pumps produced by gonococci provide a unique opportunity to understand the role of bacterial drug efflux pumps in the overall pathogenic mechanisms of bacteria during infection and how they also contribute to resistance to antibiotics used to treat infection. This review concentrates on the genetics, structure-function relationships, gene regulation systems, and overall significance of gonococcal drug efflux pumps.

17.2 Types and Substrate Profiles of Gonococcal Drug Efflux Pumps

In contrast to other bacteria that express a wide number and type of drug efflux pumps (the reader is directed to other chapters in this book for the vast repertoire of efflux pumps produced by bacteria), gonococci produce much fewer drug efflux pumps (Fig. 17.1; summarized in [6]). These pumps belong to the resistance-nodulation-cell division (RND) superfamily (MtrCDE), the major facilitator superfamily (MFS) (FarAB-MtrE), the ATP-binding cassette (ABC) transporter superfamily (MacAB-MtrE), and the multidrug toxic compound extrusion (MATE) family (NorM); see other reviews [1, 6] for a description of the discovery of these efflux pumps. Recently, a fifth gonococcal efflux pump, the MtrF protein first described by Veal and Shafer [7], which belongs to the AbgT family of transporters that typically behave as importers, was designated as an efflux pump by virtue of its ability to export sulfonamides [8]. Finally, some (rare) clinical isolates have been reported to harbor the *mef* gene [9], which encodes a pump that exports macrolides and appears to have been acquired from *Acinetobacter junii*.

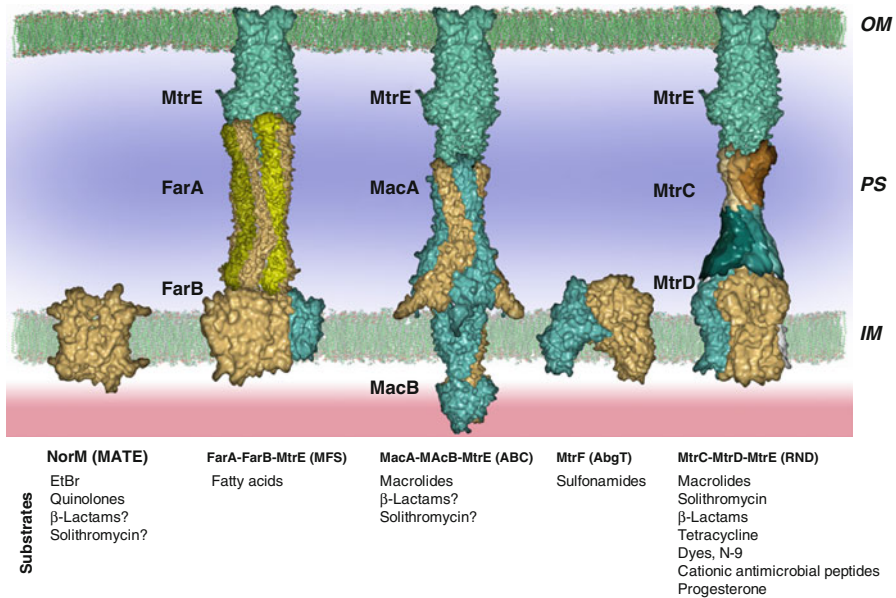


Fig. 17.1 Membrane organization of drug efflux pumps in gonococci. The proposed membrane organization and class of the known drug efflux pumps common to gonococcal strains (NorM, FarAB-MtrE, MacAB-MtrE, MtrF, and MtrCDE) as well as their substrates are depicted. MtrE most likely acts as an outer membrane channel protein for three of the pumps. MtrF possibly also acts as an accessory protein for the MtrCDE efflux pump. The crystallized NorM (PDB code: 5C6P), MtrF (4R1I), MtrD (2DHH), and MtrE (4MT0) from gonococci are shown. For MacA, MacB, FarA, FarB, and MtrC, the crystallized homologues MacA (3FPP), MacB (2HYD), EmrD (2GFP), EmrA (4TKO), and MexA (1VF7) are shown. *OM* outer membrane, *PS* periplasmic space, *IM* inner membrane. The ratio of monomeric unit number for the MtrC, MtrD, and MtrE proteins are 2:1:1, respectively, as defined earlier [25]

With respect to efflux of antibiotics, it is now recognized that the MtrCDE efflux pump can export structurally diverse hydrophobic antibiotics, β-lactams (including penicillin, nafcillin, and extended-spectrum cephalosporins) and tetracycline [5, 10–12], while the NorM pump exports quinolones [13] and the MacAB-MtrE pump exports macrolides [14]. The newly described MtrF pump recognizes sulfonamides and is proposed to be used by gonococci to export antimetabolites that accumulate within the cytosol [8]. The MtrCDE and FarAB-MtrE efflux pumps also recognize host (human and mouse)-derived antimicrobials, including cationic antimicrobial peptides (such as human cathelicidin LL-37 and murine cathelin-related antimicrobial peptide CRAMP), bile salts and progesterone (MtrCDE) [15, 16], and long-chain fatty acids (FarAB-MtrE) [17] that often bathe mucosal surfaces infected by gonococci. A functionally intact MtrCDE pump, but not FarAB-MtrE pump, is required for long-term colonization by gonococci of the lower genital tract of female mice [18], suggesting that its capacity to export host antimicrobials that pass the outer membrane helps this pathogen to establish an infection. Importantly, overproduction of the MtrCDE pump due to *cis*- or *trans*-acting regulatory mutations

(see below) cannot only increase the resistance to antimicrobials but can also increase gonococcal fitness during experimental infection of the lower genital tract of female mice [16, 19]. In total, the cumulative evidence supports the notion that the MtrCDE efflux pump is a virulence factor. Interestingly, this pump also recognizes nonionic detergents such as the spermicide nonoxynol-9, and its use over the past six decades may have inadvertently selected for gonococcal mutants that overproduce this pump [20]. The MacAB-MtrE efflux pump exports macrolides (e.g., azithromycin and erythromycin) [14]. The NorM efflux pump behaves as a Na⁺-dependent transporter [21] and exports compounds harboring a quaternary ammonium on an aromatic ring (e.g., acriflavine, berberine, ethidium bromide, and 2-*N*-methyllellipticinium) as well as ciprofloxacin and norfloxacin to the periplasmic space [13]. It is unknown if these latter two efflux pumps recognize and export host-derived antimicrobials.

17.3 Structural Features of Gonococcal Drug Efflux Pumps

Structural data on proteins that function as drug transporters or critical components of efflux pumps contribute to basic knowledge that can facilitate or advance studies on new drug development (e.g., efflux pump inhibitors (EPIs)) or alternative therapies and prevention strategies (e.g., vaccine development). With respect to gonococci, critical structural information is now available regarding two proteins (MtrD and MtrE) [22, 23] of the MtrCDE efflux pump as well as the MtrF inner membrane protein; as described above, MtrF appears to be an independent efflux pump that recognizes sulfonamides [8] and, possibly, acts as an accessory protein of the MtrCDE pump [7]. It is important to stress that, while structures have been solved for proteins that are highly similar to MtrD, MtrE, and MtrF, the information for the gonococcal proteins emphasizes that “one structure does not fit all” and that critical differences can exist between homologous proteins. These differences, as well as the potential ramifications of such, are discussed below.

17.3.1 *Crystal Structure of the MtrD Inner Membrane Efflux Pump Transporter*

The gonococcal MtrD inner membrane transporter protein of strain PID332, which is 11 amino acids shorter at the C-terminus in comparison with FA19 MtrD, was crystallized using vapor diffusion [24]. This membrane protein exists as a trimer linked to a hexamer of the periplasmic adapter MtrC, which is in turn linked to a trimer of the MtrE outer membrane channel protein [25]. At the amino acid sequence and substrate recognition levels, MtrD shares many, but not all, features of other transporters in the RND efflux pump family produced by Gram-negative bacteria.

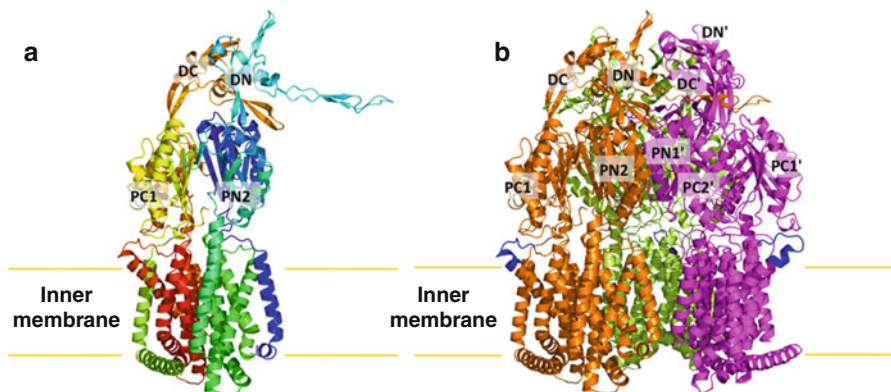


Fig. 17.2 Structure of the MtrD inner membrane transporter. (a) Ribbon diagram of a protomer of MtrD viewed in the membrane plane. The molecule is colored using a rainbow gradient from the N-terminus (*blue*) to the C-terminus (*red*). Sub-domains DN, DC, PN2, PC1, and PC2 are labeled. The location of PN1 is behind PN2, PC1, and PC2. (b) Ribbon diagram of the MtrD trimer viewed in the membrane plane. Each subunit of MtrD is labeled with a different color. Residues 917–927 (only found in MtrD) forming the upper portion of TM9 and the loop connecting TM9 and TM10 are in blue color (This figure has been reproduced from [22])

The crystal structure of the full-length PID 332 MtrD multidrug efflux pump transporter protein (114 kDa) was determined to a resolution of 3.53 Å (Protein Data Bank [PDB] code: 4MT1) [22]. The final model included 97% of the amino acids (residues 2–493 and 508–1,040) (Fig. 17.2a). The structure of MtrD is closer to the conformation of the “access” protomer of AcrB of *Escherichia coli* [26]. However, superimposition of these two structures results in a high root-mean-square deviation (RMSD) of 7.6 Å over 1,000 C α atoms, suggesting that there are significant differences between these two transporters. MtrD assembles as a 125-Å-long and 95-Å-wide homotrimer (Fig. 17.2b). Each protomer comprises 12 transmembrane helices (TM1–TM12). Like other RND transporters, the N-terminal (TM1–TM6) and C-terminal (TM7–TM12) halves of MtrD are related by a pseudo-twofold symmetry. A large periplasmic domain is created by two extensive periplasmic loops connecting TM1 with TM2 and TM7 with TM8, respectively. As in AcrB [26–30] and MexB of *Pseudomonas aeruginosa* [31], this periplasmic domain can be divided into six sub-domains: PN1, PN2, PC1, PC2, DN, and DC. Sub-domains PN1, PN2, PC1, and PC2 form the pore domain, with PN1 making up the central pore and stabilizing the trimeric organization. However, sub-domains DN and DC contribute to form the docking domain, presumably interacting with the outer membrane channel MtrE (structural information for MtrE is described below). The trimeric MtrD structure suggests that sub-domains PN2, PC1, and PC2 are located at the outermost core of the periplasmic domain, facing the periplasm. Sub-domains PC1 and PC2 also form an external cleft, and this cleft is open in the MtrD structure (Fig. 17.2). Based on the co-crystal structure of

CusBA [32, 33] of the CusCBA tripartite efflux complex of *E. coli* [34–39], the upper regions of PN2, PC1, PC2, and sub-domains DN and DC should directly interact with the MtrC membrane fusion protein to form a functional adaptor-transporter complex.

Structures of AcrB in complex with a variety of substrates [26, 29, 30] have identified that the periplasmic cleft of the pump forms several mini-binding pockets within the extensive, large periplasmic multidrug binding site. This site is supposed to play a predominant role in the selection of drugs for export. Protein sequence alignment reveals that many of the amino acids forming the large periplasmic binding site of AcrB are conserved with MexB and MtrD, indicating that these three multidrug efflux pumps may have a similar substrate binding profile for drug recognition. These conserved amino acids in MtrD are made up of several charged and polar residues, such as Ser79, Ser134, Arg174, Asp272, Glu669, and Arg714, and aromatic residues, such as phenylalanine at 136, 176, 610, 612, and 623. In addition, a flexible loop is found inside the large periplasmic cleft, which forms the multidrug binding site of the pump. This flexible loop is located deep inside the cleft between sub-domains PC1 and PC2, composed of residues 608–619, and should correspond to the Phe-617 loop [29] in AcrB. The loop is highly conserved among MtrD, AcrB, and MexB. It is expected that this flexible loop is important for drug recognition and extrusion. There is a chance that this loop may shift positions during the course of the extrusion process to facilitate drug export.

Perhaps the most interesting secondary structural feature appears in TM9 of the MtrD pump [22]. In contrast to other known structures of the RND transporters, MtrD contains an extended region that protrudes into the periplasm and contributes part of the periplasmic domain (Fig. 17.2). This region (residues 917–927) comprises an α -helix extending from the upper portion of TM9 and also to the loop connecting TM9 and TM10. Protein sequence alignment suggests that these extra residues are only found in MtrD, but not in other homologous RND proteins. Therefore, this fragment should represent a unique feature of this pump that cannot be found in other RND pumps. TM9 is distinct in that it is not vertically oriented. Instead, it is inclined from the horizontal membrane plane by 54° . The spatial arrangement between the extra elongated helix and loop (upper portion of TM9) and the periplasmic cleft formed between PC1 and PC2 suggests that these extra structural features may help the pump to transport its substrates more effectively from the outer leaflet of the inner membrane to the multidrug binding site at the periplasmic domain.

Drug export by RND multidrug transporters is proton motive force dependent [40]. Based on the crystal structure of MtrD, it is expected that the charged residues Asp405 and Asp406 of TM4 and Lys948 of TM10 are important for forming the proton-relay network of the pump. These residues are supposed to undergo protonation and deprotonation within the transport cycle. The involvement of these charged amino acids in proton translocation was supported by a previous study showing mutations of these residues inhibit proton translocation [41]. In turn, the MtrE outer

membrane channel protein is blocked and unable to dissociate from the MtrCDE tripartite efflux complex [41].

17.3.2 Crystal Structure of the MtrE Outer Membrane Channel Protein

The gonococcal MtrE protein serves as the outer membrane protein channel for the MtrCDE efflux pump, and most likely also for the MacAB and FarAB efflux pumps, that facilitates exit of antimicrobial pump substrates from the bacterial cell (Fig. 17.1; [42]). In this respect, its function in the trimer state resembles that of other outer membrane channel proteins (e.g., OprM of *P. aeruginosa* and TolC of *E. coli*) of RND efflux pumps possessed by Gram-negative bacteria. However, important differences between MtrE vs. OprM/TolC exist, and these were illuminated by structural work [23] and are summarized below.

The gonococcal MtrE protein of strain FA136 was used for crystallization using vapor diffusion. Protein sequence alignment suggests that FA136 MtrE and FA19 MtrE are nearly identical, except for the amino acid residues between 178 and 197. In FA136 MtrE, these residues are NAVRIAVQGRDRFRRRPAPA. The corresponding residues in FA19 MtrE are KLSELRYKAGVISAVLRQQ. As mentioned in the following paragraph, these residues form a loop region between the periplasmic helices H3 and H4 of FA136 MtrE. It was later found that the protein sequence of FA136 MtrE was in error, possibly due to a DNA sequencing error when the sequence of FA136 *mtrE* gene was determined two decades ago. Nonetheless, the crystal structure of the FA136 MtrE outer membrane channel protein (prepared from expression of a synthetic gene based on the FA136 sequence) was determined to a resolution of 3.29 Å (PDB code: 4MT0) [23]. The final model comprises 99% of the total amino acids (residues 1–445) (Fig. 17.3). The MtrE and OprM channels share 40% protein sequence identity. However, superimposition of the structure of MtrE with that of OprM [41] results in an RMSD of 18.2 Å over 445 C α atoms, suggesting a highly significant difference in the overall tertiary structures between these two channel proteins. An important insight gained from the MtrE structural data is the recognition of the presence of two, short surface-exposed domains (6 per trimer) that are antigenic and now under investigation as part of the newly rejuvenated vaccine effort (see below); these surface-exposed domains represent residues 93–98 and 300–310 [23].

Like OprM [43], TolC [44], CusC [36, 37], and CmeC [45], MtrE exists as a homotrimer that forms a ~130-Å-long α/β barrel (Fig. 17.3). Each subunit of MtrE contains four β -strands (contributing to the 12-stranded outer membrane β -barrel) and eight α -helices (forming the elongated periplasmic α -barrel): H1 (41–53), H2 (55–78), S1 (85–92), S2 (99–114), H3 (119–182), H4 (188–226), H5 (254–260), H6 (262–284), S3 (288–299), S4 (311–320), H7 (328–395), and H8 (400–435). These four β -strands (S1, S2, S3, and S4) constitute the β -barrel domain and are organized in an antiparallel fashion, spanning the outer membrane. In contrast, the

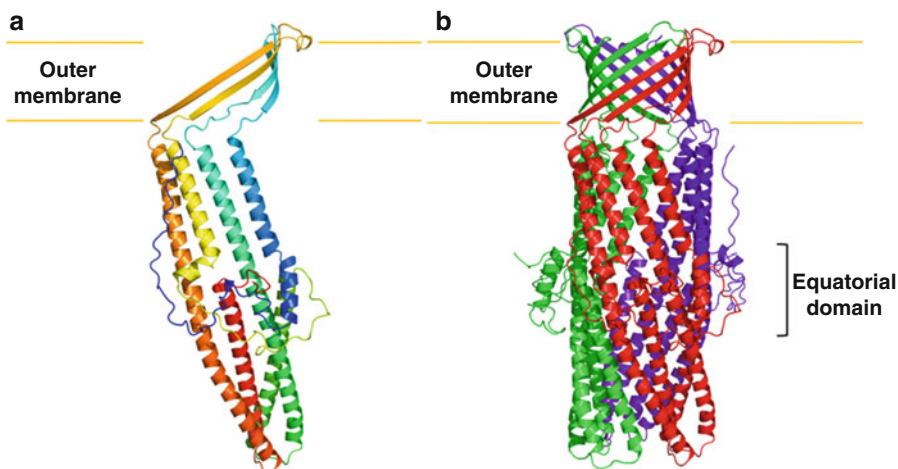


Fig. 17.3 Structure of the MtrE outer membrane channel protein. (a) Ribbon diagram of a protomer of MtrE viewed in the membrane plane. The molecule is colored using a rainbow gradient from the N-terminus (*blue*) to the C-terminus (*red*). (b) Ribbon diagram of the MtrE trimer viewed in the membrane plane. Each subunit of MtrE is labeled with a different color (This figure has been reproduced from [23])

elongated periplasmic tunnel of MtrE contains six α -helices. Similar to the structure of TolC [44], two long α -helices (H3 and H7) are found to extend across the entire length of the periplasmic α -helical tunnel. The α -helical tunnel of MtrE also includes two pairs of shorter α -helices (H2 and H4) and (H6 and H8). These two pairs of shorter α -helices stack end to end to form pseudo-continuous helices, which contribute coiled-coil interactions with the two long helices. The equatorial domain of MtrE is composed of two α -helices (H1, H5), and the remaining elements at this domain are mostly unstructured. The periplasmic tunnel of MtrE is ~ 100 Å long with an outermost diameter of ~ 35 Å at the tip of the tunnel.

To date, all available structures of outer membrane channel proteins, such as TolC [44], OprM [43], CusC [36, 37], and CmeC [45], indicate that the interior surfaces of these channels are highly electronegative. However, MtrE is distinct in that its internal surface does not have extensive positively or negatively charged patches. On the contrary, the charge distribution of the outside surface of MtrE is very similar to other outer membrane channels, in which the outside surfaces of all these channels have no extensive charged patches. In view of the crystal structure of MtrE, the internal surface of the protein forms a continuous channel. This channel is completely open and fully accessible through both the periplasmic end and outer membrane surface, suggesting that the MtrE channel is at its open conformational state. Most of the available structures of outer membrane channels, including TolC, OprM, CusC, and CmeC, are closed at one or both sides [42–45]. The widest section of the channel is located at the surface of the outer membrane, with the internal diameter of ~ 22 Å. The volume of the continuous channel formed by the internal surface of the MtrE trimer is $\sim 45,000$ Å³ [23].

The architecture of the interior of the MtrE channel is quite similar to that of TolC [42, 46]. An aspartate ring is found at the periplasmic entrance of the interior of the MtrE channel. Each protomer of MtrE contributes Asp402 and Asp405 to form two concentric circles of negative charges in the inner cavity of the trimeric MtrE channel [23]. Thus, this interior aspartate ring is composed of six aspartate residues. In TolC, the corresponding aspartate ring creates a selectivity gate for this channel and this ring can be blocked by large cations. The internal diameter of the MtrE aspartate ring is ~ 12 Å, which creates the narrowest region of the tunnel. It is likely that this aspartate ring is responsible for the selectivity of the channel, similar to the case of TolC [46]. Indeed, it has been demonstrated that the aspartate ring of MtrE can be blocked by the large positively charged hexamminecobalt (III) complex [41]. During the course of substrate import or export, the aspartate ring may still need to dilate more and increase its internal diameter to allow for the passage of substrates through the channel. Although the structure indicates that MtrE is capable of opening this channel by itself, it has been suggested that the dilation and constriction of the aspartate ring may be controlled by the MtrC periplasmic membrane fusion protein [25]. In addition, it has been observed that the MtrE channel is able to allow the large vancomycin molecule to enter the cell but only does so in response to the binding of the membrane fusion protein MtrC adaptor [25], presumably enhancing the degree of dilation of the MtrE channel. It appears that the opening and closing of the MtrE channel may be induced by the change in conformation of the MtrC protein, which propagates the progressive motion of the MtrD multidrug efflux pump within the transport cycle to the MtrE channel. As MtrD is a proton motive force-dependent pump, this may imply that active proton translocation within the MtrD efflux pump provides the energy to open and close the MtrE channel.

As mentioned earlier, it is likely that the sequence of the reported FA136 *mtrE* gene (and the originally predicted MtrE amino acid sequence) was incorrect due to a DNA sequencing error; we thank V. Bavro (University of Birmingham, United Kingdom) for bringing this to our attention. We have started crystallizing the FA19 MtrE channel protein. Hopefully, its structure will be determined and reported in the near future so that a more complete understanding of MtrE can be obtained.

17.3.3 Crystal Structure of MtrF: A Novel Drug Efflux Pump Protein

The MtrF transporter protein was recently implicated as a novel efflux pump protein possessed by gonococci that has the capacity to export antimetabolites and sulfonamide drugs [8]. The capacity of MtrF to export sulfonamide drugs was first realized when recombinant MtrF expressed in *E. coli* reduced levels of bacterial susceptibility to sulfonamides, which was verified by biochemical and genetic

studies using mutant versions of the *mtrF* gene. At the amino acid level, the 522-amino acid MtrF protein belongs to the *p*-aminobenzoyl-glutamate transporter (AbgT) family that typically serve as importers [7]. MtrF shares 38 % identity with *E. coli* AbgT. Interestingly, AbgT has been shown to enable uptake of the folate catabolite *p*-aminobenzoyl-glutamate, but work with MtrF indicates that it can export the folate metabolite paraminobenzoic acid (PABA). Circumstantial genetic evidence also suggests that MtrF can serve as an accessory protein for the MtrCDE efflux system when the latter is overexpressed in strains of gonococci harboring regulatory mutations ([7]; see below).

We have determined the crystal structure of gonococcal MtrF (strain FA19) to a resolution of 3.95 Å (PDB code: 4R1I) (Fig. 17.4) [8]. Crystals of MtrF belong to space group P6₅. Two molecules of MtrF, which assemble as a dimer, are found in the asymmetric unit. Superimposition of these two MtrF molecules gives an RMSD of 0.5 Å over 506 C α atoms, indicating that their conformations are nearly identical to each other. The crystal structure of the MtrF dimer reveals a bowl-shaped concave aqueous basin, approximately 75 Å tall, 80 Å wide, and 50 Å thick. The rim of the basin is as large as 45 Å in diameter and penetrates into the inner leaflet of the cytoplasmic membrane by approximately 25 Å. This deep basin probably allows aqueous solution to reach until the midpoint of the membrane bilayer.

Overall, the secondary structure of the MtrF dimer is very similar to that of *Alcanivorax borkumensis* YdaH [47], which also belongs to the AbgT family of transporters. A pairwise superimposition of the MtrF dimer onto YdaH results in overall RMSD of approximately 4 Å. Each molecule of MtrF comprises nine

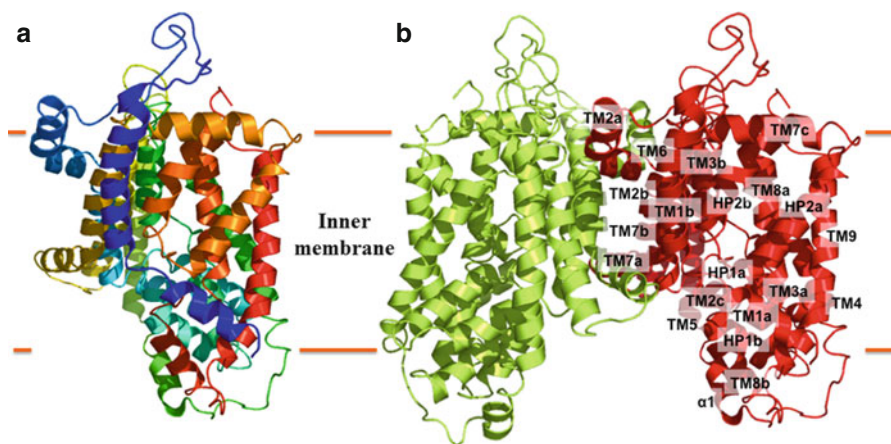


Fig. 17.4 Structure of the MtrF efflux pump. (a) Ribbon diagram of a protomer of MtrF viewed in the inner membrane. The molecule is colored using a rainbow gradient from the N-terminus (blue) to the C-terminus (red). (b) Ribbon diagram of a dimer of MtrF viewed in the inner membrane. The right subunit of the dimer is colored red, whereas the left subunit is colored green. The MtrF dimer forms a bowl-shaped structure with a concave aqueous basin facing the intracellular solution (This figure has been reproduced from [8, 48] (with permission from the publisher))

transmembrane α -helices and two helical hairpins: TM1 (a (12–22) and b (26–47)), TM2 (a (78–92), b (94–112), and c (114–125)), HP1 (a (128–145) and b (147–164)), TM3 (a (168–182) and b (191–205)), TM4 (218–240), TM5 (269–292), TM6 (310–334), TM7 (a (341–353), b (356–374), and c (376–391)), HP2 (a (396–413) and b (417–434)), TM8 (a (438–451) and b (462–471)), and TM9 (480–506). In addition to HP1 and HP2, which are only long enough to span half of the membrane, the transmembrane helices TM1, TM2, TM3, TM7, and TM8 are broken into multiple segments within the membrane. These segmented loops allow the transporter to form an internal cavity within the membrane [8, 48].

Each protomer of MtrF contains a relatively small periplasmic domain. This domain is made up of two long loops formed between TMs 1 and 2 and TMs 5 and 6, respectively. Below the inner leaflet of the membrane, a small cytoplasmic domain links TMs 4 and 5 together. This domain is comprised by a relatively long random loop and helix (α 1). The MtrF dimer can also be divided into inner and outer core regions. The inner core, comprising TM1, TM2, TM5, TM6, and TM7, creates a frame-like housing for the inner core and contributes to the dimerization domain. Involved in this dimerization interface are TM1b, TM2a, TM2b, TM6, TM7a, and TM7b, as well as the corresponding segments from the next subunit.

The outer core comprises TM3, TM4, TM8, TM9, HP1, and HP2. Like YdaH [47], the helices and hairpins of the outer core of MtrF form a tunnel spanning approximately from the middle of the inner membrane up to the periplasm. Interestingly, this tunnel is connected to the cytoplasm via an opening in the basin formed by the loop regions of HP1, HP2, TM3, and TM8. Importantly, several conserved residues, including Asp193, Trp420, Pro438, and Asp449, line the wall of the tunnel. It is expected that these residues may play an important role for the function of this transporter. It has also been shown that MtrF is able to export PABA and sulfonamide drugs from the bacterial cell and that the conserved residues Asp193, Trp420, Pro438, and Asp449 are important for this function [8, 48]. Accordingly, these conserved residues are critical for the function of the *N. gonorrhoeae* MtrF efflux pump and strongly support the idea that MtrF act as efflux pumps and participate in exporting the metabolite PABA and sulfonamide antimetabolites from the cell.

17.4 Gonococcal Efflux Pump Genes

Of the five efflux pumps associated with all gonococci strains thus far examined [1, 6, 8], participating proteins of three are encoded by genes organized in an operon (i.e., *mtrCDE*, *farAB*, and *macAB*). The multiple transferable resistance (*mtr*) locus, which includes *mtrF*, was first identified by Pan and Spratt [49] and then further elucidated by Hagman et al. [10, 24], Delahay et al. [42], and Veal and Shafer [7] by conventional cloning and sequencing technologies. The other gonococcal efflux pump systems [13, 14, 17] were identified by mining the FA1090 genome sequence that was made available online by D. Dyer and colleagues in 1998 (<http://www.genome.ou.edu>).

17.4.1 *The mtrCDE Efflux Pump Operon*

The genetics of the *mtr* system have been extensively studied for the past two decades, and it is the best understood gonococcal efflux pump system to date. Mtr has its origin from a phenotype identified by Maness and Sparling in 1973 [50] when they isolated a spontaneous gonococcal mutant that exhibited increased resistance to multiple, structurally diverse antimicrobial hydrophobic compounds. It was originally thought that the associated, but then unknown, mutation decreased cell envelope permeability by overproducing a 52-kDa outer membrane protein and increasing the degree of peptidoglycan cross-linking [51]. However, subsequent cloning/sequencing experiments in the mid-1990s [10, 49] showed that the Mtr phenotype was due to mutations within the coding region or in a promoter upstream of a gene encoding a transcriptional repressor (MtrR) in the TetR/QacR family [52]. The *mtrR* gene is positioned 250 base pairs (bp) upstream and transcriptionally divergent from the *mtrCDE* operon that encodes the tripartite MtrCDE efflux pump similar to other RND-type pumps (e.g., AcrAB-TolC) of Gram-negative bacteria [10, 24, 42, 49]. As described above, like other RND efflux pumps, the three proteins that form the pump are a cytoplasmic (inner) membrane transporter (MtrD), a membrane fusion protein (also called periplasmic adapter protein; MtrC), and an outer membrane channel protein (MtrE) (Fig. 17.1); as mentioned above, it is most likely that MtrE also serves as the outer membrane channel protein for the FarAB and MacAB efflux pumps (Fig. 17.1; [14, 17] and W. M. Shafer et al. unpublished).

Directly or indirectly, other proteins or outer membrane components also participate in efflux mediated by the MtrCDE pump. In this respect, Veal and Shafer [7] suggested that MtrF could serve as an accessory protein for high levels of antimicrobial resistance mediated by the MtrCDE efflux system. Additionally, energy supplied by the TonB-ExbBD system is needed for inducible antimicrobial resistance mediated by MtrCDE [53] and this induction process requires the participation of a transcriptional activator termed MtrA (see below). Lipooligosaccharide structure is also important in the function of the MtrCDE efflux pump in strains overexpressing *mtrCDE* because a deep rough lipooligosaccharide mutant expressing a core oligosaccharide that was severely truncated was unable to express high-level resistance to substrates of the pump [54].

While the *mtr* locus is highly conserved in gonococci, clinical isolates frequently contain mutations within the *mtrR* structural gene or the associated promoter that increase *mtrCDE* expression and resistance to antimicrobial substrates (see below). The *cis*- or *trans*-acting regulatory mutations are of clinical and biological significance since they appear to be important in the ability of gonococci to resist certain clinically useful antibiotics as well as provide a fitness advantage over wild-type gonococci (see below). Interestingly, a minority of strains, some of which also have *mtrR* mutations, contain small deletions in the *mtrC* or *mtrD* genes that increase gonococcal susceptibility to antimicrobials recognized by the MtrCDE efflux pump [55]. These so-called *env* (*envelope*) mutants were originally identified by the Sparling laboratory in the mid-1970s [56]. The advantage such strains might have

during infection is unclear, but their ability to donate antimicrobial resistance due to *mtrR* mutations is of interest since such genetic exchange may help in the emergence of natural gonococcal variants with decreased susceptibility to efflux pump substrates during infection.

17.4.2 The *mtrF* Gene

The *mtrF* gene was discovered by Veal and Shafer [7] during a study of a gonococcal strain that displayed an *env* phenotype (hypersusceptibility to antimicrobials recognized by the MtrCDE efflux pump) yet contained a wild-type *mtrCDE* operon. Subsequent cloning and sequencing efforts showed that the clinical isolate contained a small deletion in an open reading frame downstream of *mtrR* that would encode a protein within the AbgT family of transporters [8]; the structural aspects of MtrF and AbgT proteins are described above. Interestingly, loss of *mtrF* seemed to have an impact on gonococcal resistance to antimicrobials recognized by the MtrCDE efflux pump only when *mtrCDE* was overexpressed due to *cis*-acting mutations (described below) and the gonococcal strain had a mutant version of the PorB1b porin as opposed to wild-type PorB1b or PorB1a [7]; the latter phenomenon highlights the interplay between membrane components for gonococcal resistance to antimicrobials [57]. In this context, amino acid replacements in PorB1b at position 120 alone (Gly120Lys) or positions 120 and 121 (Gly120Asp/Ala121Asp) are frequently observed in gonococcal strains expressing decreased susceptibility to β -lactam antibiotics [1, 57]. Thus, it was proposed that MtrF serves as an accessory protein used by the MtrCDE efflux pump when gonococci are confronted by high levels of antimicrobials [7]. While this original hypothesis may still be valid, more recent work [8] implicates MtrF as an independent efflux pump that exports sulfonamide antimetabolite drugs. As a member of the AbgT family of proteins that normally import substrates, the illumination of MtrF efflux activity defines a new family of bacterial efflux pumps; continued work is necessary to better understand its roles in gonococcal physiology and metabolism.

17.4.3 The *farAB* Efflux Pump Operon

The *farAB* operon in gonococci was identified by mining the FA1090 genome sequence (<http://www.genome.ou.edu>) for open reading frames that would encode drug efflux pumps similar to those possessed by other Gram-negative bacteria [17]; the *farAB* gene products are similar to the *emrAB*-encoding efflux pump system of *E. coli*. Expression of *farAB* is regulated by both *cis*- and *trans*-acting systems (see below). As mentioned above, the FarAB efflux pump in gonococci most likely uses MtrE as its outer membrane protein channel (Fig. 17.1) to export long-chain fatty acids such as palmitic and oleic acid. Fecal-derived fatty acids can have potent anti-gonococcal activity under

laboratory conditions [58], and their efflux by the FarAB-MtrE pump may help gonococci survive during rectal infections, which are frequently asymptomatic [1].

17.4.4 The macAB Efflux Pump Operon

The *macA* and *macB* genes are organized in an operon, and their predicted products are similar to the MacA and MacB proteins produced by other Gram-negative bacteria [14]. The MacAB efflux pump most likely uses MtrE as its outer membrane protein channel, belongs to the ABC transporter superfamily, and exports macrolides and, at least to some extent, possibly also β -lactams and the fluoroketolide solithromycin (Fig. 17.1; [5]). The promoter that drives expression of *macAB* in strain FA19 has a point mutation in the -10 hexamer sequence that dampens expression of this operon [14]; this mutation has been observed in other gonococcal strains (Reimche et al. unpublished observations). Recent evidence (Kandler et al. manuscript in preparation) suggests that *macAB* expression is enhanced by a response regulator (MisR) of a two-component regulatory system (MisRS).

17.4.5 The norM Efflux Pump Gene

The *norM* gene encodes the NorM efflux pump [13], which belongs to the MATE family and exports compounds harboring a quaternary ammonium on an aromatic ring, quinolones, and, at least to some extent, possibly also β -lactams and solithromycin [5]. The *norM* gene is located upstream of open reading frames that have been provisionally annotated (<http://www.genome.ou.edu>) as encoding the MurB enzyme involved in *N*-acetylmuramic acid biosynthesis and a TetR-like repressor. Additional work is needed, however, to confirm these functions and if the three genes in this region are co-transcribed by a common promoter. Point mutations within the *norM* ribosome binding site and a putative -35 promoter hexamer sequence have been identified, and these likely modulate expression of *norM* and, as a consequence, levels of NorM [13].

17.5 Transcriptional Regulation of Gonococcal Efflux Pump Genes

Discoveries of bacterial efflux pumps were often made prior to the availability of whole genome sequences and were facilitated by the isolation of mutants that expressed decreased susceptibility to antimicrobials [52, 59]. These resistance-conferring mutations frequently mapped to a gene that encoded a DNA-binding protein that would normally dampen expression of a closely linked gene or operon

encoding efflux pump proteins. As an example of this and with respect to gonococci, Pan and Spratt [49] discovered the *mtr* locus in strain CH95 by identifying a mutation in *mtrR*, which encodes a transcriptional repressor (MtrR) of the *mtrCDE* efflux pump-encoding operon (see below). It is now recognized that, in addition to mutations that impact the activity of DNA-binding proteins, *cis*-acting mutations located within promoter sequences can have stronger influences on efflux pump gene expression and levels of bacterial susceptibility to antimicrobials recognized by the cognate efflux pump. Against this background, the complexities of *cis*- and *trans*-acting transcriptional factors that modulate gonococcal efflux pump gene expression and bacterial susceptibility to antimicrobials are described below.

17.5.1 *Cis-Acting Factors that Regulate Efflux Pump Genes*

Cis-acting regulatory mutations can have a profound impact on the expression of gonococcal genes encoding efflux pumps and can be responsible for levels of antimicrobial resistance due to export mechanisms; the most comprehensively studied *cis*-acting regulatory system involves expression of the *mtrCDE*-encoded pump (Fig. 17.5). In this respect, point mutations, deletions, or insertions in the nucleotide sequences between *mtrR* and *mtrCDE* can provide gonococci with higher levels of antimicrobial resistance than mutations within the *mtrR*-coding region. For instance, a single-bp deletion within the 13-bp inverted repeat element localized in the *mtrR* promoter (Fig. 17.5) can significantly enhance transcription of *mtrCDE* [10, 60]. While not all macrolide-resistant clinical gonococcal isolates have this bp deletion [61], it was frequently observed in a panel of strains isolated in Seattle, USA, obtained from men who have sex with men during an outbreak of macrolide-resistant gonorrhea [62]. Some clinical isolates have a dinucleotide insertion within this inverted repeat [63]. In either case, the optimal 17-bp spacing between the -10 and -35 elements is disrupted, and this significantly reduces *mtrR* transcription to nearly undetectable levels. This mechanism, however, cannot be the sole reason why such strains express high-level antimicrobial resistance since their level of *mtrCDE* expression is greater than strains with *mtrR* loss-of-function or null mutations [10]. Instead, because the promoters for *mtrR* and *mtrCDE* transcription overlap and are divergent, it is more likely that the mutations enhance RNA polymerase interactions with the *mtrCDE* promoter.

A limited number of gonococcal strains (e.g., MS11 and WHO L) have a point mutation (C→T) located 120 nucleotides upstream of the *mtrC* translational start codon (*mtr*₁₂₀), changing the sequence at this region from TATAAC to TATAAT [19, 64] and thereby generating a consensus -10 element (Fig. 17.5) [65]. This new -10 element acts as a stronger promoter (*mtr*₁₂₀) for *mtrCDE* transcription than the wild-type promoter, and its use results in high levels of *mtrCDE* expression and resistance to hydrophobic agents, as well as enhanced *in vivo* fitness [19]; importantly, this new promoter is outside of the control by MtrR, which exerts repressive activity on the wild-type promoter. The identification of this novel promoter that enhanced

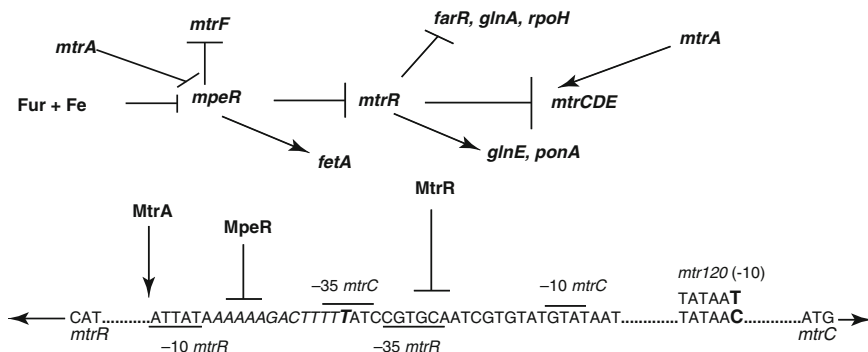


Fig. 17.5 Regulatory features of the *mtr* locus. Shown are the *cis*- and *trans*-acting regulatory elements that control expression of the *mtrCDE* operon as well as “off-target” genes (see text for details). Negative regulation of genes by DNA-binding proteins is shown by the *barred lines*, while transcriptional activation of genes by proteins is shown with *arrows*. Sites of binding by MtrR, MtrA, and MpeR are shown. The main mutations impacting promoters are the single-base pair (bp) deletion in the *mtrR* promoter region, which is designated by the larger font T nucleotide as is the mutation that generates the novel *mtr*₁₂₀ promoter. The nucleotide sequence shown represents the *mtrCDE* coding strand; the translational start codons for *mtrR* and *mtrC* are shown at the ends of the sequence. The 13-bp inverted sequence within the *mtrR* promoter is shown in italics (This figure has been modified from [1])

transcription of *mtrCDE* independently of MtrR repression allowed Ohneck et al. [66] to study the genome-wide transcriptional influence that might result when the MtrCDE pump is produced at elevated levels. Such a study had not been possible previously since all other mutants that overexpressed *mtrCDE* did so in a background where MtrR would be absent making it difficult to know if any observed transcriptional changes were influenced by this transcriptional regulator. In brief, use of the *mtr*₁₂₀ promoter (Fig. 17.5) in an otherwise wild-type background resulted in increased expression of 13 genes outside of the *mtr* locus. Included in this set of genes was *ccp*, and its overexpression due to elevated expression of *mtrCDE* was correlated with decreased susceptibility of gonococci to peroxides. Thus, it is important to realize that overexpression of bacterial efflux pumps can have unexpected consequences on gene expression that could influence metabolism and resistance to antimicrobials that are not pump substrates.

Some gonococcal strains, notably those from an outbreak of azithromycin-resistant gonorrhea in Kansas City, USA, in 1999 [67], contain a 153-bp insertion in the *mtrR*-*mtrCDE* intervening region. These isolates expressed resistance to MtrCDE efflux pump substrates. Interestingly, this (or a closely related) insertion, identified as a Correia element (CE) [68], can be found within this region of the *mtr* locus in many, but not all, strains of *Neisseria meningitidis* (meningococci) [69, 70]. Some serogroup Y meningococcal strains also have a tandemly linked IS1301 sequence [69]. In meningococci, the presence of the Correia element dampened *mtrCDE* expression as a result of providing a binding site for integration host factor (IHF) and a new site for posttranscriptional processing of the *mtrC* transcript [71];

IHF is also important in regulating the *farAB* efflux pump operon (see below). It is unclear how the Correia element and perhaps more importantly the IHF-binding site and IHF function in gonococci since hydrophobic agent resistance is elevated in strains bearing the Correia element sequence. Nevertheless, the presence (albeit rare) of the Correia element at this site in gonococci suggests that horizontal gene transfer occurred between meningococci and gonococci. If true, this event emphasizes the importance of recombination in generating diversity in clinical strains of both pathogens.

Expression of *norM* and *macAB* genes is also modified by *cis*-acting control elements that were defined by mutations in gonococcal clinical isolates. The presence of point mutations in the -35 hexamer of the *norM* promoter (C to T) or in a putative ribosome binding site (A to G; reference 70 for consensus sequence) can enhance gonococcal resistance to all substrates of the NorM pump [13]. A point mutation in the -10 hexamer of the *macAB* promoter (G to T) has been identified that increases expression of *macAB* and levels of macrolide resistance in gonococci [14]. Together with other mutations that impact antimicrobial susceptibility levels in gonococci, the presence of these *cis*-acting mutations could influence the efficacy of antimicrobial therapy, particularly for strains with susceptibilities near the minimal inhibitory concentration (MIC) breakpoint for the relevant antimicrobials. Like the *mtr*₁₂₀ mutation for *mtrCDE*, the point mutations found to regulate *norM* and *macAB* alter the sequence of their respective promoter elements to be closer to consensus [65]. Thus, it appears that point mutations within bacterial promoters can upregulate gene expression by enhancing promoter recognition by RNA polymerase outside of the control of transcriptional regulators or, in the case of *norM*, may also increase translation through improved ribosomal recognition of the *norM* transcript.

17.5.2 Trans-Acting Factors that Regulate Efflux Pump Genes

A number of DNA-binding proteins directly or indirectly control expression of genes encoding structural proteins of bacterial drug efflux pumps [51, 58], including those of gonococci [12]. Their function proved crucial in early studies dealing with the identification of cognate efflux pump-encoding genes [52]. For gonococci, the DNA-binding proteins that control *mtrCDE*, *mtrF*, and *farAB* expression are especially understood and are the subject of discussion below.

DNA-binding proteins that control *mtrCDE* and *mtrF* gene expression

Expression of genes within the *mtr* locus is directly or indirectly controlled negatively or positively by a number of DNA-binding proteins (summarized in Fig. 17.5 and references [1, 6, 12]). Central to this regulation is MtrR, which was first described by Pan and Spratt [49] that acts to repress *mtrCDE* expression by binding as two homodimers to the *mtrCDE* promoter region [72, 73]. Studies on antimicrobial-resistant clinical isolates have shown that such strains often contain mutations that cause radical amino acid replacements in the helix-turn-helix (HTH)

motif (residues 32–53) of MtrR that decrease its binding to target DNA sequences [72] while other mutations map outside of the HTH region that might alter dimer formation or drug interactions. All of these loss-of-function mutations can enhance *mtrCDE* transcription two- to threefold and, depending on the substrate, increase resistance to antimicrobials by four- to tenfold [11, 74]. It is important to emphasize that, because loss-of-function mutations of *mtrR* have been observed in gonococcal clinical isolates, their occurrence is relevant for considering antibiotic treatment regimens used worldwide.

In addition to its prominent regulatory action on the *mtrCDE* promoter, MtrR can directly or indirectly control expression of a number (>65) of other genes at different phases of growth [75]. Cumulative results from genetic and transcriptional profiling studies identified MtrR as a global regulatory protein. Briefly, MtrR has been shown to control genes involved in the generalized stress response (*rpoH*) [75], peptidoglycan biosynthesis (*ponA*) [76], amino acid biosynthesis (*glnA* and *glnE*) [77, 78], polyamine uptake (*potF* and *potH*) [75], and regulation of a regulator (*farR*) of the *farAB* efflux pump operon [79]; the capacity of MtrR to activate or repress expression of a non-*mtr* related genes is shown in Fig. 17.5. We have termed these genes as being “off-target,” since MtrR is acting as a *trans*-regulator to distinguish them from the adjacent *mtrCDE* genes [77].

Expression of *mtrR* is negatively controlled by the product of the *mpeR* gene [80], which in turn is negatively controlled by Fur (ferric uptake regulator) + iron [81] and the MtrA transcriptional activator of *mtrCDE* (Fig. 17.5; [1]). This result suggests that levels of antimicrobial resistance and *in vivo* fitness due to the MtrCDE efflux pump could be influenced by the availability of free iron during infection or inducing agents that work through MtrA (see below). Accordingly, it is important to realize that MIC values obtained for gonococci grown on iron-rich laboratory media may not reflect absolute susceptibility of the test strain during infection and these MICs might accordingly be higher *in vivo*. MpeR was discovered during a search of the FA1090 genome sequence for regulators [81], in addition to MtrR, that might control expression of *mtrF* [82]. The capacity of Fur + iron to repress *mpeR* and the ability of MpeR to repress *mtrR* likely explain why *mtrCDE* is maximally expressed late in growth when levels of free iron would be low [81]. It has been shown that MpeR activates expression of *fetA* [83], which encodes a single-component TonB-dependent receptor that allows the gonococcus to acquire iron from enterobactin-like siderophores produced by enteric bacteria [84]. Taken together, these findings show that MpeR plays important roles in both drug efflux and iron acquisition by gonococci, emphasizing the need to consider regulators of efflux genes in a larger context that includes general concepts of bacterial physiology and pathogenesis.

Gonococci, like other bacteria, can activate transcription of drug efflux pump-encoding genes when confronted with antimicrobial substrates. In this instance, transcriptional activators typically bind “inducers” (pump substrates) and then more effectively interact with DNA sequences that are usually located near the efflux pump-encoding genes. The best example of this phenomenon in gonococci is the action of MtrA [20], a member of the AraC family of transcriptional regulators such

as Rob, which is critical in activating transcription of efflux pump-encoding genes ([85]; see [1, 86] for summary reviews). MtrA was discovered during experiments that evaluated whether growth of the gonococci in sublethal level of substrates recognized by the MtrC-MtrD-MtrE efflux pump could enhance bacterial resistance to such antimicrobials. A panel of strains was tested and some were found to increase their resistance to Triton X-100 by an MtrA-dependent manner when incubated overnight in sublethal levels of Triton X-100 [20]. Interestingly, many gonococcal isolates [20, 87], including strain FA1090 [20], contain an 11-bp deletion within *mtrA* and are unable to transcriptionally activate *mtrCDE* in the presence of an inducer. This MtrA-dependent inducible resistance property requires energy delivered from the TonB-ExbBD system. MtrA has been purified, and its DNA-binding action upstream of the *mtrCDE* promoter has been defined [88]). Consistent with Triton X-100 being an inducer, its presence increased MtrA binding to its target DNA and even outcompeted MtrR in the latter's binding to the *mtrCDE* promoter (Fig. 17.5; [88]). This result provides a mechanism for upregulation of *mtrCDE* expression in the presence of an inducer. MtrA, like MtrR and MpeR, has global regulatory action and can modulate expression of a number of genes outside of the *mtr* locus (W. M. Shafer et al. unpublished) including direct or indirect repression of *mpeR* (Fig. 17.5; [1]).

DNA-binding proteins that regulate the *farAB* operon The *farAB* operon in gonococci is subject to both direct and indirect regulation by DNA-binding proteins. First, it is subject to direct repression by FarR, a member of the MarR family of transcriptional regulators, by its interaction with the *farAB* promoter [79]. This binding is enhanced by the DNA-binding/DNA-bending action of IHF [89]. Second, but indirectly, transcription of *farAB* is controlled by MtrR as this protein binds to the *farR* promoter resulting in dampening of *farR* transcription [79]. Thus, with opposing consequences, MtrR can directly and indirectly regulate levels of two gonococcal efflux pump operon systems, which results in repression of one operon (*mtrCDE*) and activation of another (*farAB*).

17.6 Efflux Pumps as Contributors to Gonococcal Antimicrobial Resistance

From a historical perspective (summarized in [1, 6]), the first line of evidence that an efflux pump could impact levels of gonococcal susceptibility to antimicrobials can be gleaned from the early work of the Sparling laboratory conducted in the 1970s [50, 90]. Their studies on antimicrobial resistance showed that the then undefined *mtr* mutation, now known to be the single-bp deletion in the inverted repeat sequence of the *mtrR* promoter region (Fig. 17.5), could increase gonococcal resistance (two- to eightfold) to relatively hydrophobic antibiotics such as erythromycin, rifampicin, chloramphenicol, and tetracycline, as well as benzylpenicillin. This increased antimicrobial resistance afforded to gonococci by overexpression of the MtrCDE efflux pump was not by itself clinically significant. However, subsequent

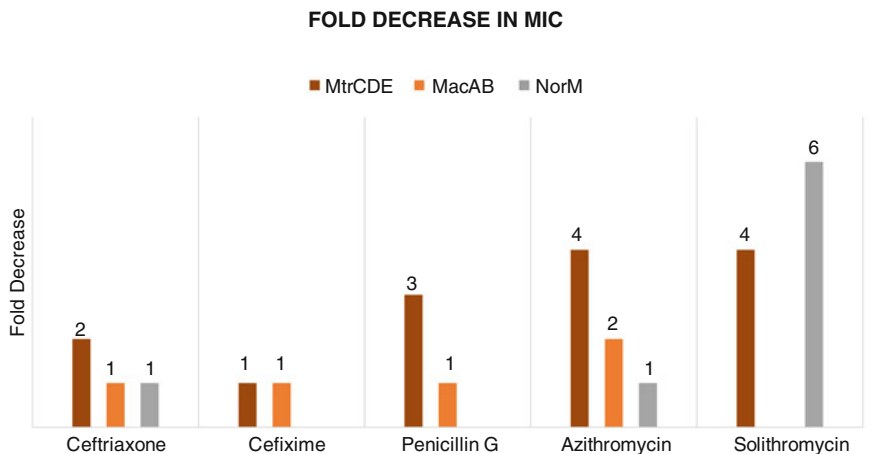


Fig. 17.6 Loss of gonococcal efflux pumps MtrCDE, MacAB-MtrE, and NorM increases bacterial susceptibility to antimicrobials. The impact of loss of individual pumps on gonococcal (strain HO41) susceptibility to selected antimicrobials is shown as fold decrease in MIC relative to parental strain HO41 (as designated by the numeral over each bar). A onefold difference represents a one dilution change in the susceptibility of the mutant compared to parent strain HO41, and the absence of a bar signifies that loss of the respective pump had no impact on the MIC value (The figure was adapted from data reported by Golparian et al. [5])

work by the Sparling group, and later by the Nicholas laboratory, showed that co-resident mutations that altered the structure of the β -lactam targets penicillin-binding protein 2 (PBP2) and PBP1 [91, 92], as well as the porin PorB1b [57, 93], which decrease the influx of antimicrobials, would act with the *mtr* mutation to provide full penicillin resistance.

In hindsight, the findings of Sparling and coworkers were remarkable in that we now can better appreciate that, while overexpression of bacterial drug efflux pumps may not provide bacteria with clinical resistance to antimicrobials, it can in conjunction with other mutations be of clinical importance. Work with the *mtr* system in gonococcal clinical isolates such as strain FA6140, which caused the penicillin-resistant (non- β -lactamase) outbreak of gonorrhea in North Carolina, USA, in 1983 [94], and others is an example of such cooperativity of mutations in providing clinically relevant levels of resistance to antimicrobial drugs. Veal et al. [11] showed that loss of the MtrCDE efflux pump rendered this isolate clinically sensitive to penicillin. FA6140 contains the single-bp deletion in the *mtrR* promoter as well as mutations in *ponA* (encoding PBP1), *penA* (encoding PBP2), and *porB1b* (encoding PorB1b). Zarantonelli et al. [63] further showed that *mtrR* promoter mutations that increased expression of *mtrCDE* provided gonococci with low, but clinically relevant, levels of azithromycin resistance and that this could be reversed by genetic inactivation of the *mtrCDE* efflux pump operon. More recently, Golparian et al. [5] showed that loss of the *mtrCDE* efflux pump system in clinical isolates, including the infamous HO41 strain from Japan [95], which caused the first reported case of high-level ceftriaxone-resistant gonorrhea, reversed both penicillin and azithromycin resistance; resistance to extended-spectrum cephalosporins (cefixime and ceftri-

axone) was reduced by up to twofold and solithromycin by fourfold (Fig. 17.6; [5]). In addition to the contributions of the MtrCDE pump, the NorM and MacAB-MtrE efflux pumps can contribute to gonococcal resistance levels to certain antibiotics. Based on results from MIC determination assays that compared wild-type and efflux pump-deficient mutant strains, NorM antimicrobial substrates seem to include quinolones, possibly β -lactams, and solithromycin, while MacAB-MtrE substrates include macrolides and solithromycin (Fig. 17.6; [5, 13, 14]). Taken together, it is now clear that the presence and overexpression of gonococcal efflux pumps can contribute significantly to bacterial resistance to antimicrobial drugs used previously (e.g., penicillin), presently (azithromycin and cephalosporins) or in the future (solithromycin) in gonorrhea treatment regimens. As is discussed below, efforts that target these pumps in drug therapy or vaccines may help in future treatment protocols or prevention strategies.

17.7 Gonococcal Efflux Pumps as Virulence Factors

In the absence of classical antibiotics, does expression of an efflux pump provide an advantage for gonococci (or any bacteria) during infection? This is a critical question because contributions of efflux pumps to bacterial survival prior to appearance of symptoms may influence proliferation and dissemination of the invading pathogen before antibiotic therapy begins. Results from studies on the MtrCDE efflux pump suggest that this is the case since it was required for gonococcal survival during experimental infection of the lower genital tract of female mice [18]. Loss of this efflux pump increases gonococcal susceptibility to intracellular killing by mouse and human polymorphonuclear leukocytes (A. E. Jerse et al. unpublished) and mouse macrophages, but not human ME180 cervical epithelial cells (D' Andrea and Shafer, unpublished). Importantly, differential expression of the *mtrCDE* operon by the transcriptional regulators MtrA and MtrR can modulate fitness levels of gonococci in the murine infection model [16, 19]. Briefly, loss of MtrA in strain FA19 decreased *in vivo* fitness of gonococci by ca. 1,000-fold, while null mutations in *mtrR* or promoter mutations (e.g., single-bp deletion in the *mtrR* promoter or the novel *mtr*₁₂₀ promoter) increased *in vivo* fitness by ca. 100- and 1,000-fold, respectively. In total, these results with the murine experimental infection model suggest that the MtrCDE efflux pump is important for gonococci to survive *in vivo* when challenged by mediators of innate host defense [16, 19]. This capacity to resist mediators of the innate host defense may be important during human infection because gonococci elicit a strong T_H17 pro-inflammatory response [96], and such a response is often characterized by enhanced production of antimicrobial peptides. The capacity of gonococci to export free long-chained fatty acids (e.g., palmitic and oleic acid) via the FarAB-MtrE efflux pump may help it survive in the rectum where concentrations of such fatty acids are elevated [58, 74]; however, direct experimental evidence is lacking since FarAB-MtrE was not required for *in vivo* survival in the genital tract of female mice, and currently, there is no animal model of rectal infection by gonococci [18].

Based on the above observations, one might ask: why did gonococci not dispense with MtrR since its loss increases antimicrobial resistance and *in vivo* fitness? It is possible that so-called “off-target” genes activated by MtrR (see above) hold the key. Thus, in the mouse infection experiments performed by Warner et al. [16], loss of MtrR afforded a fitness advantage for the first 5 days, but this advantage waned, suggesting that possession of MtrR may be advantageous at later stages of infection. One of these MtrR-activated genes that may be important *in vivo* is *glnE* [75, 78], which is of importance in *Salmonella enterica* serovar Typhimurium growth and fitness *in vivo* [97]. GlnE encodes the enzymatic regulator of glutamine synthetase and can activate or deactivate the enzyme. Since levels of glutamine are low at mucosal surfaces and within phagocytes, the ability to synthesize glutamine *in vivo* may be important for survival and maximal fitness of gonococci. A possible advantage for MtrR-producing gonococci is also suggested by work of Kunz et al. [98], which concentrated on the impact of *gyrA* and *parC* mutations on gonococcal fitness during infection; these mutations confer gonococcal resistance to fluoroquinolones. In relationship to *mtrR*, a spontaneous mutant recovered from mice infected with a strain bearing *gyrA* and *parC* mutations as well as a single-bp deletion in the *mtrR* promoter displayed increased fitness. This mutant had, surprisingly, repaired the deletion such that a wild-type *mtrR* promoter would exist, but the reason why this *in vivo* evolved mutant had a competitive advantage over the parental strain remains unclear [98]. Additional work that uses transcriptional profiling and whole genome sequencing to identify additional mutations that might contribute to this phenotype and construction of mutant strains in this genetic background is needed to pinpoint the mechanism(s) by which this mutant strain has a competitive advantage *in vivo*.

17.8 Targeting Gonococcal Efflux Pumps for Drug or Vaccine Development

Given the importance of the MtrCDE efflux pump in resistance to classical antibiotics and host-derived antimicrobials, would it be beneficial to target this pump for future drug development (e.g., EPIs) or vaccine development? Using EPIs, coadministered with antimicrobials, could allow for the return of previously used and relatively inexpensive antibiotics (e.g., penicillin [5]) and/or render gonococci more susceptible to host-derived antimicrobials, thereby promoting natural clearance by the host. Furthermore, if the EPI has the identical binding site on the efflux pump as the antimicrobial administered, a target modification would be disadvantageous for the bacteria, and this would likely also suppress the resistance development. Below, two recent approaches that target the MtrCDE efflux pump are described.

Efforts to develop safe and effective EPIs have to date failed largely due to toxicity issues [99]. It is important to emphasize, however, that these EPIs targeted inner membrane transporters (e.g., MtrD-like proteins) and cross-recognition by eukaryotic importers/exporters may explain the observed toxicity. A proposed alternative

strategy [100] would be to target the membrane fusion/adaptor component (MtrC-like proteins) of the RND pumps as their structures do not have eukaryotic homologues. Indeed, one of our laboratories (E. W. Yu) is developing peptides that bind MtrC and prevent assembly of the Mtr pump *in vitro*. Follow-up studies are in progress to determine if they can sensitize gonococci to antimicrobial drugs such as β -lactams and macrolides.

After decades of relative futility in developing a vaccine to prevent gonorrhoea, renewed efforts are now underway by several international groups to develop a gonorrhoea vaccine [101]. To a large extent, these efforts have been driven by the emerging problem of antimicrobial resistance and the fear of untreatable infections in the future. One of us (A. E. Jerse) is targeting MtrE as it has two short immunogenic peptide sequences exposed on the surface (see Sect. 17.3.2 above; [23]). The value of targeting MtrE is that it is highly conserved among gonococci, stably produced, and most likely used by at least three efflux pumps (MtrCDE, FarAB-MtrE, and MacAB-MtrE) to export antimicrobials (Fig. 17.1). In preliminary experiments, female mice immunized with recombinant MtrE lacking the first two N-terminal amino acids in conjunction with CpG adjuvant were found to clear gonococci faster than mice immunized with MtrE + cholera toxin adjuvant or CpG adjuvant alone. An important consideration herein is that the CpG adjuvant would drive a T_H1 response thought to be important in generating protective immunity against gonococci [96, 101]. Although these studies are in their infancy, the initial results give hope that MtrE could be part of a future vaccine to at least protect at-risk populations from gonorrhoea. It should also be noted that anti-MtrE antibodies might also sensitize gonococci to antimicrobials (e.g., β -lactams and macrolides) recognized by the MtrCDE efflux pump system, which would in theory counteract efflux-dependent resistance mechanisms.

17.9 Concluding Remarks

As has been recently emphasized [1, 3], the public health problem of multidrug-resistant gonococcal strains is becoming more severe, and strains expressing clinical resistance to extended-spectrum cephalosporins would significantly impact therapeutic options in controlling gonorrhoea worldwide. In the absence of new antimicrobials that recognize novel targets, this problem will only worsen, and new efforts in the drug development field are required. Based on the considerable progress made in the past 20 years on gonococcal efflux pumps with respect to their identification, structure, function, gene regulation, and important contributions to antimicrobial resistance and virulence, which have been the major topics covered herein, we posit that targeting efflux pumps for drug/vaccine development has merit and should be part of the current anti-infective pipeline to combat gonorrhoea. Finally, given the importance of efflux pumps in antimicrobial resistance in gonococci, these basic research efforts over the past decades have helped to contribute to

a better understanding of the genetics, gene regulation, structure, and biologic functions of bacterial efflux pumps in general.

Acknowledgments We thank all past and present members of our laboratories and our collaborators who have contributed significantly to the work reviewed herein. We are grateful to V. Bavro for communicating information regarding the *mtrE* sequence of *N. gonorrhoeae*. This work was supported by NIH grants R37 AI021150 (W.M.S.), R01 AI114629 (E.W.Y.), R01 AI42053 (A.E.J.), and U19 AI113170 (A.E.J.) and funds from the Örebro County Council Research Committee and the Foundation for Medical Research at Örebro University Hospital, Sweden (M.U.). W.M.S. is the recipient of a Senior Research Career Scientist Award from the Biomedical Laboratory Research and Development Service of the Department of Veterans Affairs. The contents of this chapter do not represent the views of the Department of Veterans Affairs, the National Institutes of Health, or the US government.

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