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Elizabeth A. Ohneck, Emory University
Maira Goytia, Emory University
Corinne Loughlin, Emory University
Sandeep J. Joseph, Emory University
Timothy Read, Emory University
Ann E. Jerse, Uniformed Services University of the Health Sciences
William Shafer, Emory University

Journal Title: Antimicrobial Agents and Chemotherapy
Volume: Volume 59, Number 1
Publisher: American Society for Microbiology | 2015-01-01, Pages 724-726
Type of Work: Article | Final Publisher PDF
Publisher DOI: 10.1128/AAC.04148-14
Permanent URL: https://pid.emory.edu/ark:/25593/pqsw3

Final published version: http://dx.doi.org/10.1128/AAC.04148-14

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Accessed October 14, 2017 7:26 PM EDT
Overproduction of the MtrCDE Efflux Pump in Neisseria gonorrhoeae Produces Unexpected Changes in Cellular Transcription Patterns

Elizabeth A. Ohneck,a Maira Goytia,a Corinne E. Rouquette-Loughlin,a Sandeep J. Joseph,b Timothy D. Read,b Ann E. Jerse,c William M. Shafera,d

Departments of Microbiology and Immunologya and Medicine,b Emory University School of Medicine, Atlanta, Georgia, USA; Department of Microbiology and Immunology, F. Edward Herbert School of Medicine, Uniformed Services University of the Health Sciences, Bethesda, Maryland, USA;c; Laboratories of Bacterial Pathogenesis, VA Medical Center, Decatur, Georgia, USAd

The global consequence of drug efflux gene overexpression in bacteria has not been specifically analyzed because strains showing high-level expression typically have mutations in genes encoding regulatory proteins that control other genes. Results from a transcriptional profiling study performed with a strain of Neisseria gonorrhoeae that is capable of high-level transcription of the mtrCDE efflux pump operon independently of control by cognate regulatory proteins revealed that its overexpression has ramifications for systems other than drug efflux.

Bacteria use efflux pumps to resist the action of antibiotics during treatment of infection or to survive in the presence of antimicrobials in their environment (1). The level of expression of efflux pump-encoding genes is modulated by a complex regulatory system of repressors and activators, but derepression due to mutation in repressor-encoding genes or their promoters can significantly decrease bacterial susceptibility to antimicrobials (2). As an example, mutation in the mtrR repressor gene of Neisseria gonorrhoeae enhanced transcription of the mtrCDE-encoded efflux pump and resulted in both increased resistance of gonococci to structurally diverse antimicrobials (3–5) and greater in vivo fitness in an experimental mouse model of lower genital tract infection (6, 7). MtrR also has more global action, however, as it directly or indirectly impacts expression of >65 genes involved in diverse systems such as peptidoglycan synthesis, pilin secretion, the general stress response, and glutamine metabolism (8–10).

The question asked in this study was this: does overexpression of a drug efflux pump per se have a potential impact on bacteria? Heretofore, this question could not be addressed because bacteria that overexpress efflux pump genes typically contain mutations that impact cognate gene regulators (e.g., MtrR), which would make interpretation of results difficult. Taking advantage of a unique promoter that drives high-level expression of mtrCDE independently of MtrR control (11) (as well as activation by MtrA [12]), we performed a transcriptional profiling study. Here, we provide data that support the hypothesis that overexpression of a bacterial efflux system may have unexpected global ramifications with respect to microbial physiology.

Bacterial strains, culture conditions, and antimicrobial testing. N. gonorrhoeae strains FA19 and FA19mtr120 were the major strains used (Table 1). “mtr120” signifies a single nucleotide change (C to T) 120 nucleotides upstream of mtrCDE (7) that generates a new promoter for mtrCDE transcription (11) and increases gonococcal resistance to diverse antimicrobials (Table 1), including host defense antimicrobials (7, 13). Transformants of these strains bearing an insertionally inactivated ccpR gene due to the presence of the nonpolar aphA-3 cassette were constructed by previously described methods (12). Gonococci were routinely grown as non-piliated, opacity-negative variants on gonococcal medium base (GCB) agar or in broth, each with defined supplements I and II, as previously described (4). The MIC of antimicrobials recognized by the MtrCDE efflux pump was determined by agar dilution (4). To test gonococcal susceptibility to peroxides, a microtiter plate assay was used. Briefly, this involved exposing 10⁶ gonococci in GCB broth to various concentrations of hydrogen peroxide (H₂O₂) or tert-butylhydroperoxide (tBuOOH) at 37°C for 45 min before spotting 5 μl onto GCB agar plates for assessment of viability; H₂O₂ and tBuOOH are not substrates of the MtrCDE pump (unpublished observations).

RNA-seq and qRT-PCR studies. A transcriptional-profilin-study using transcriptome sequencing (RNA-seq) and three independent RNA samples prepared from broth-grown, late-logarithmic cultures of isogenic strains FA19 and FA19mtr120 was performed using previously described methods (14). We defined differentially expressed genes as having (i) a fold change value of ≥2, (ii) a total read number larger than 5, and (iii) a Bonferroni-corrected P value of ≤0.05. For quantitative reverse transcriptase PCR (qRT-PCR) analysis of gene expression, RNA samples from RNA-seq experiments were used to synthesize cDNA using random hexamers. Transcripts of the genes of interest (ccpR and rpsS15) were quantified using qRT-PCR and oligonucleotide primers (see Table S1 in the supplemental material) essentially as described previously (14); rpsS15 was used as a reference for normalization of the results.

Global gene expression consequences of overexpression of mtrCDE. As expected from previous studies (7, 11), RNA-seq
analysis revealed that expression of mtrC, mtrD, and mtrE was highly upregulated in strain FA19mtr120 compared to wild-type strain FA19, which, based on earlier reports (4, 5, 9), served as an internal control for the results of the transcriptome comparison (Table 1); this upregulation was confirmed by qRT-PCR (see Table S1 in the supplemental material). Importantly, analysis of the RNA-seq results revealed changes in the expression levels of 13 non-mtr genes which were widely distributed across the genome (Fig. 1) and represented a variety of functional classes (Table 1) based on annotation of the FA1090 genome (www.genome.ou.edu). Only one of these non-mtr genes (ccpR) was overexpressed in strain FA19mtr120. Proteins encoded by the 13 underexpressed genes included four hypothetical proteins; a protease (encoded by clpP); a periplasmic peptidase (sohB); a stationary-phase-survival protein (surE); an ATP-dependent RNA helicase (NGO1248); a putative SAM-dependent methyltransferase (NGO1481); and a putative integrative membrane protein (NGO0585).

**TABLE 1 Transcriptional response of gonococci to overexpression of mtrCDE efflux pump operon**

<table>
<thead>
<tr>
<th>Gene and category</th>
<th>Common name</th>
<th>Fold change</th>
<th>Functional classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upregulated in the presence of the mtr120 mutation</td>
<td>mtrE 4.44</td>
<td>Mtr efflux pump protein component: outer membrane channel protein (MtrE)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>mtrD 5.63</td>
<td>Mtr efflux pump protein component: RND family transporter (MtrD)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>mtrC 5.42</td>
<td>Mtr efflux pump protein component: periplasmic fusion protein (MtrC)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ccpR 2.52</td>
<td>Cytochrome c peroxidase</td>
<td></td>
</tr>
<tr>
<td>Downregulated in the presence of the mtr120 mutation</td>
<td>Ngo0218 -15.02</td>
<td>Hypothetical</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ngo1085 -2.02</td>
<td>Hypothetical integral membrane protein</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ngo1083 -2.03</td>
<td>ATP-dependent Clp protease subunit</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ngo1082 -2.14</td>
<td>Hypothetical</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ngo1084 -2.05</td>
<td>ATP-dependent RNA helicase</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ngo1086 -2.14</td>
<td>Stationary-phase-survival protein</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ngo1087 -2.72</td>
<td>Periplasmic peptidase, S49 family</td>
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<tr>
<td></td>
<td>Ngo1088 -2.53</td>
<td>Hypothetical</td>
<td></td>
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<td></td>
<td>Ngo1089 -3.56</td>
<td>FadR/GntR family transcriptional regulator</td>
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<td></td>
<td>Ngo1090 -2.67</td>
<td>Mtr efflux pump accessory protein</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ngo1091 -4.59</td>
<td>Putative SAM-dependent methyltransferase</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ngo1092 -4.83</td>
<td>Protein translocase channel subunit</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ngo1093 -3.06</td>
<td>Transmembrane transporter, tellurium resistance</td>
<td></td>
</tr>
</tbody>
</table>

RND, resistance-nodulation-division; SAM, S-adenosylmethionine.

FIG 1 Shown are chromosomal-map positions of genes differentially expressed in strain FA19mtr120 compared to wild-type strain FA19 as identified by RNA-seq analysis (Table 1). The circular map is from the annotated FA1090 genome (www.genome.ou.edu). Upregulated genes are shown in green, while downregulated genes are shown in red.
phase-associated survival protein (surE); a transcriptional regulator (NGO1360) involved in modulating expression of glutamate metabolism genes in meningococci (15) and located just downstream of the upregulated mtrCDE operon; an ATP-RNA helicase (NGO0650); a component of the protein translocating system (secE); a tellurium resistance-associated protein (terC); and a putative methytransferase (NGO1481).

We selected ccpR, which encodes cytochrome C peroxidase (16), as a model gene to further test if overexpression of mtrCDE could have distal effects on gonococcal gene expression; ccpR was also chosen as it may contribute to gonococcal resistance to oxidants given the multiple ways gonococci resist peroxides (16,17). By qRT-PCR analysis, we confirmed expression changes occur is unclear but should be considered in drug efflux studies.

Nucleotide sequence accession numbers. The complete data set can be accessed through GEO accession number GSE47048 and SRA accession number SRA079863.

ACKNOWLEDGMENTS

We thank Jacqueline Balthazar for excellent technical assistance and Yaranah Zalucki for critical reading of early versions of the paper.

This work was supported by NIH grants AI021150-29 (W.M.S.), AI42053 (A.E.J.), AI096788 (T.D.R., D. Dean, and R. Selden), and AI031496-22 (to P. F. Sparling, University of North Carolina) and a VA Merit Review Grant to W.M.S. W.M.S. is the recipient of a Senior Research Career Scientist Award from the VA Medical Research Service.

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