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Phasevarions Mediate Epigenetic Regulation of Antimicrobial Susceptibility in *Neisseria meningitidis*

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Phase variation is a common feature of host-adapted bacterial pathogens such as *Neisseria meningitidis*. Recently, we reported that this rapid on/off switching of gene expression occurs in DNA methyltransferases, altering expression in multiple genes via changes in global methylation. In the current study, we compared MIC values of strains with ModA11, ModA12, and ModD1 phasevarions, revealing MIC differences due to ModA11 and ModA12 switching, with a ModA11_OFF strain showing 4-fold reduced susceptibilities to ceftazidime and ciprofloxacin.

Neisseria meningitidis can cause potentially fatal systemic disease, and prompt diagnosis and antimicrobial intervention are essential for favorable clinical outcomes. Resistance to several antibiotics, including penicillin (1), tetracycline (2), sulfonamides (3), quinolones (4), and rifampin (5), has been reported.

Phase variation, the high-frequency on/off switching of gene expression, is a common feature of host-adapted bacterial pathogens such as *N. meningitidis*. In recent studies, we reported that phase-variable expression can occur in N^6-adenosine DNA methyltransferases (Mod) as a result of the hypermutation of simple DNA repeats within the open reading frame. This leads to the reversible loss/gain of repeat units, which in turn leads to frameshift mutations and on/off switching of Mod expression. The resulting changes in global DNA methylation in the on/off states cause global changes in gene regulation and have been defined as phasevarions (phase-variable regulons (6, 7). In pathogenic *Neisseria* species, two distinct classes of Mod proteins, ModA (8) and ModD (9), have been studied in detail. The genes encoding each of these Mod proteins have different alleles that are based on amino acid differences in their DNA recognition domains. Each Mod allele recognizes and methylates a different DNA sequence and affects the expression of a different set of genes. For example, the on/off switching of ModD1 affects the susceptibility of *N. meningitidis* to hydrogen peroxide (9), and the on/off switching of ModA13 affects the susceptibility of *Neisseria gonorrhoeae* to the antimicrobial detergent Triton X-100 (8). The precise mechanisms by which Mod methylation of DNA regulates differential gene transcription remain unclear, but the phenomenon of epigenetic regulation in bacteria has been well described, and DNA methylation can alter the interaction of regulatory proteins with DNA-binding sites (10).

ModA11, ModA12, and ModD1 are among the most common phase-variable DNA methyltransferases found in *N. meningitidis*. Here, MIC assays were used to examine whether ModA11, ModA12, and ModD1 are involved in the susceptibility of *N. meningitidis* to a range of antibiotics. The antibiotics tested included those that are currently in clinical use to treat patients with meningococcal disease and their contacts and those to which colonizing *N. meningitidis* may be exposed during the treatment of other infections (11).

The *N. meningitidis* MC58 (ModA11), B6116/77 (ModA12), and M0579 (ModD1) Mod ON and *mod*::*kan* knockout strains were used to compare relative antibiotic susceptibilities. Each of these strains contains a different DNA methyltransferase, which recognizes and methylates a distinct DNA sequence (K. L. Seib, F. E.-C. Jen, A. Tan, A. L. Scott, R. Kumar, P. M. Power, L. T. Chen, H. L. Wu, A. H.-J. Wang, M. Boitano, T. A. Clark, J. Korlach, D. N. Rao, and M. P. Jennings, submitted for publication). The expression statuses of *mod* genes in the cultures used were confirmed by GeneScan fragment length analysis (ABI, Life Technology), as previously described (8, 9). The MICs of strains MC58 *modA11*_ON 1R (locked-ON *modA11*, i.e., contains 1 repeat and is unable to phase vary to OFF), B6116/77 modA12_ON (77% ON), and M0579 *modD1*_ON (90% ON) were compared to those of MC58 *modA11*::*kan*, B6116/77 *modA12*::*kan*, and M0579 *modD1*::*kan* knockout mutants (8, 9), respectively. Meningococcal strains were grown under iron limitation conditions (RPMI [Gibco] with 10 μM deferoxamine mesylate [Desferal; Sigma]) in order to mimic conditions found in the host. Since DNA methyltransferases affect gene regulation by means of competition or interaction of regulators with methylated DNA, it is important that phasevarion experiments be performed under conditions in which regulators relevant to *in vivo* infection are active. Bacteria were then incubated in brain heart infusion (BHI) broth containing 1 of 13 antibiotics that act as inhibitors for cell wall, protein, or DNA synthesis (see Table 1 for the antibiotics used). MICs were measured by broth microdilution in three replicated experiments as described previously (11).

The MIC results (Table 1) revealed that the ModA11- and ModA12-containing strains were more sensitive to several antibiotics when the Mod protein was expressed and DNA was methylated (Seib et al., submitted for publication). The ModA11_ON 1R strain was 2-fold more susceptible to cloxacillin, doxycycline, and nalidixic acid and 4-fold more susceptible to ceftazidime and ciprofloxacin than was the *modA11*::*kan* strain. The ModA12_ON strain was also 2-fold more sensitive to cephalothin, cloxacillin, and rifampin than was the *modA12*::*kan* strain. Differences in the

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TABLE 1 MICs of 13 antibiotics for *N. meningitidis* strains containing ModA11, ModA12, and ModD1 DNA methyltransferases

Antibiotic (breakpoints)[a]	MIC (mg/liter) for strain[b]: M0579 *modD1*_ON	M0579 *modD1*::*kan*	B6116/77 *modA12*_ON	B6116/77 *modA12*::*kan*	MC58 *modA11*_ON 1R	MC58 *modA11*::*kan*	MIC_{50} (range) (mg/liter)[c]
Ampicillin (S ≤ 0.12; R > 1)	0.063	0.063	0.063	0.063	0.063	0.063	0.06 (≤.007 to 1)
Carbenicillin	0.031	0.031	0.063	0.063	0.063	0.063	
Cefotaxime (S ≤ 0.12; R > 0.12)	0.003	0.003	0.003	0.003	0.003	0.003	0.003 (≤.0015 to 0.03)
Ceftazidime	0.031	0.031	0.063	0.063	***0.031 (0.032)***	***0.125 (0.094)***	
Cephalothin	0.156	0.156	*0.313 (0.38)*	*0.625 (0.75)*	0.313 (0.38)	0.313 (0.38)	
Ciprofloxacin (S ≤ 0.03; R > 0.06)	0.006 (0.004)	0.006 (0.004)	0.006 (0.004)	0.006 (0.004)	***0.006 (0.004)***	***0.025 (0.015)***	0.003 (≤.0015 to 0.25)
Cloxacillin	1.250	1.250	*1.250 (1.5)*	*2.500 (3.0)*	*1.250 (1.5)*	*2.500 (3.0)*	
Doxycycline	0.750	0.750	0.375 (0.38)	0.375 (0.38)	*0.375 (0.38)*	*0.750 (0.75)*	0.5 (0.12 to 2)
Nalidixic acid	1.250	1.250	1.250 (1.0)	1.250 (1.0)	*1.250 (1.0)*	*2.500 (2.0)*	1 (0.5 to >64)
Penicillin G	0.031	0.031	0.063	0.063	0.063	0.063	0.06 (≤.007 to 1)
Piperacillin	0.031	0.031	0.031	0.031	0.031	0.031	
Rifampin (S ≤ 0.25; R >0.25)	0.063	0.063	*0.125 (0.2)*	*0.250 (0.4)*	0.125 (0.2)	0.125 (0.2)	0.12 (≤0.007 to >256)
Tetracycline (S ≤ 1; R > 2)	0.625	0.625	0.625	0.625	0.313	0.313	1 (0.5 to >64)

[a] EUCAST clinical MIC breakpoints (mg/ml) based on agar diffusion susceptibility tests. For ciprofloxacin and rifampin, breakpoints apply only to use in the prophylaxis of meningococcal disease (13). S, sensitive; R, resistant.

[b] MICs are reported as the last concentration at which turbidity was observed. Each antibiotic and strain were tested in three independent experiments, with identical MICs observed for each strain/antibiotic pair between experiments. The numbers in italic type show a 2-fold difference, and the numbers in bold italic type show a 4-fold difference. Numbers in parentheses are the MICs measured using Liofilchem MIC test strips and Oxoid M.I.C.Evaluator strips.

[c] MICs at which 50% of the 442 meningococcal isolates tested (124 strains for doxycycline) were inhibited using broth microdilution and agar dilution susceptibility tests. MIC ranges from this study are shown in parentheses (12).

MICs were confirmed by Liofilchem MIC test strips and Oxoid M.I.C.Evaluator strips (according to the manufacturers' instructions) (Table 1). On the other hand, there was no MIC difference observed between the ModD1_ON and *modD1*::*kan* strains. This indicates that ModD1 has no effect on antibiotic susceptibility and that the strategy of comparing the wild-type ON strains with a *mod* knockout containing a kanamycin resistance gene had no effect on susceptibilities to the set of 13 antibiotics tested.

These results suggest that gene regulation through DNA methylation is an additional element that may contribute to the development of antibiotic resistance under selective pressure. Although the ModA-dependent 2- and 4-fold differences observed are not expected to directly result in treatment failures, the synergy of ModA allele switching with other mutations may expand the meningococcal antibiotic resistance spectrum. A range of MICs is seen for different meningococcal isolates (12), and susceptibility/resistance breakpoints have not been defined for many antibiotics for *N. meningitidis*. However, based on information from the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (13), the decreased susceptibility for *modA*::*kan* compared to that for ModA_ON is 1 dilution from the resistance breakpoint for ciprofloxacin (ModA11) and at the breakpoint for rifampin (ModA12) (see Table 1). In the clinical setting, ciprofloxacin-resistant *N. meningitidis* strains were reported from 2 outbreaks in Delhi, India, in 2007 (14), in France in 2008 (15), and in North America in 2009 (16). Ciprofloxacin-resistant strains were observed to have point mutations in the DNA gyrase A (*gyrA*) gene reducing the susceptibility to fluoroquinolones (14). However, ModA11-dependent ciprofloxacin susceptibility is *gyrA* independent, as sequencing of the *gyrA* gene from the ModA11_ON 1R and ModA11 knockout strains showed identical sequences for these two strains. In addition, rifampin-resistant meningococci were found to have point mutations in the RNA polymerase β subunit (*rpoB*) gene (17), and a recent study showed that the loss of PorB decreased the susceptibility of *N. meningitidis* to doxycycline, cephalothin, and ceftazidime (11).

Our previous studies defined the genes regulated by ModA11, ModA12 (8), and ModD1 (9) phasevarions under the conditions used in the current study. Hundreds of genes were differentially regulated in the MC58 *modA11*::*kan* strain relative to that in the ModA11_ON strain, and 26 genes were differentially regulated in the *modA12*::*kan* strain relative to the ModA12_ON strain. However, none of these is an obvious well-characterized antibiotic resistance mechanism (i.e., *gyrA*, *rpoB*, and *porB* are not part of these phasevarions). Current studies are focused on identifying the genes within these phasevarions that are responsible for the observed reduced susceptibilities.

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