mBio - observation

High-frequency switching between ON and OFF states in the phase variable *modB* methylase impacts gonococcal azithromycin susceptibility

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Abstract

The gonococcal adenine methylase *modB*, belonging to a Type III restriction modification system, is phase variable, but both the frequency of phase variation and phenotypic impact of phase variation is largely unknown.
Here we show that *modB* switches between the ON and OFF states at a high frequency, with multiple transition events identified over the time scale of a single outbreak. Expanding our analyses, we find a clear association between *modB* status and azithromycin susceptibility. Among isolates lacking canonical 23S and *mtrR* promoter mutations, carrying *modB* in the ON configuration is strongly associated with increased azithromycin minimum inhibitory concentrations. Our findings suggest that *modB* adenine methylation controls the expression of genes modulating azithromycin susceptibility.

Importance

Despite significant progress, our current understanding of the genetic basis of antibiotic susceptibility remains incomplete. The gonococcal methylase *modB* is phase variable, meaning that it can be switched ON or OFF via contraction or expansion of a repeat tract in the gene. We find that transitions between the ON and OFF state occur at high frequency. Among strains lacking well-known resistance mutations, a functional *modB* was found to be associated with decreased susceptibility to azithromycin, a key drug used to treat gonorrhea. This finding brings us a step closer to a full understanding of the genetic underpinnings of antibiotic resistance.

Introduction

Neisseria gonorrhoeae has one of the largest known repertoires of restriction modification systems (RMS), with 13-15 systems present in individual genomes (1). The phase variable methylase *modA* has been shown to regulate the expression of genes involved in antigenicity, infection and antibiotic resistance in both *Neisseria meningitidis* and *N. gonorrhoeae (2)*. In a study of *N. meningitidis*, locking the methylase *modA11* in ON configuration resulted in significantly higher susceptibility to ciprofloxacin and third generation cephalosporins compared to a strain with *modA11* knocked out (3).

In *N. gonorrhoeae*, two adenine methylases, *modA* and *modB*, which belongs to separate Type III RMS methylases (NgoAXII and NgoAX, respectively), contain genic repeat-tracts resulting in phase variation (2). In addition, the specificity unit *hsdS* of the Type I RMS NgoAV is also phase variable, but phase variation of *hsdS* alters the target motifs that are methylated rather than turning the system ON or OFF (1). Beyond their involvement in restriction modification, these RM systems thus potentially provide the means for rapid re-arrangements of genome-wide adenine methylation (6mA) patterns and transcription. Here we investigate the frequency of phase variation in *modB* and probe its role in azithromycin susceptibility.

Results

Rapid phase variation of modB *at a microevolutionary scale*

We investigated the expansion and contraction of phase variable tracts in the Type III RMS system methylases *modA* and *modB*, in addition to the Type I RMS specificity unit *hsdS*. First, we selected 10 isolates belonging to the multilocus sequence type (ST) 9363 for nanopore sequencing, as genome-wide methylation load can be read directly from the raw sequencing data (4). For each isolate, hybrid assemblies were generated from Illumina and Oxford Nanopore data using Unicycler (5). Closed genome assemblies were generated for each, ensuring essentially error-free assembly of the RMS modules of interest. This also enabled the direct assessment of target-motif methylation load as a function of RMS status across the 10 isolates.

We found *hsdS* to be invariable in our isolates, suggesting that phase variation of the gene occurs at a relatively modest rate. The methylases *modA* and *modB* were however found to vary in phase across the 10 ST-9363 isolates. For *modA*, we did not observe a clear effect of predicted ON/OFF status on the methylation load on either of the two suggested target motifs identified previously namely GCAG*A* (1) or AGAAA (2). The gene however has a number of putative start codons which suggests that the gene might retain some functionality via other combinations of start codons and tract lengths.

In *modB*, phase variation is mediated by the contraction or expansion of a (CCCAA)n repeat tract (2). There was a clear association between the inferred ON/OFF status and corresponding methylation load on the CC*A*CC target motif (Fig. 1 a). To get a better understanding of the rate of phase variation, we determined the ON/OFF status of the gene in a larger collection of ST-9363 isolates, and mapped the status on a time-resolved phylogeny (see methods). This mapping revealed that *modB* underwent switching between the ON and OFF state at a high rate across the phylogeny, also within a single outbreak spanning less than 3.5 years of evolution (Fig. 1 b).

ModB modulates azithromycin susceptibility

Next, we investigated whether *modB* could play a role in modulating drug susceptibility. We focused on azithromycin, as this is one of two cornerstone-drugs used to treat gonorrhea, and also a drug for which genome-based prediction of susceptibility is particularly challenging (6).
From a database containing genomes sequenced at the Norwegian Institute of Public Health between 2016 and 2018, we identified a total of 911 isolates with available azithromycin minimum inhibitory concentrations (MICs) for which *modB* tract lengths could be determined. As a limited number of mutations are known to strongly impact azithromycin susceptibility, namely specific mutations in *23S* (A2059G and C2611T) and the *mtrR* promoter (-35A deletion), we excluded isolates carrying these mutations from our analyses. This left a total of 455 isolates. A chi-square analysis revealed that *modB* ON/OFF status was not randomly distributed across MIC groups ($\chi 2$ $p = 0.0008$), with *modB* ON clearly associated with higher MIC values (Fig. 2 a). The effect size of the association was relatively strong (Cohen's $d = 0.42$) and a bootstrap randomization of MIC values over *modB* status revealed that the effect size was highly significant ($p < 1E-4$, Fig. S1). The distribution of *modB* configuration across the 911 *N. gonorrhoeae* genomes demonstrated that the observed association is independent of population structure (Fig. 2 b).

Conclusions and further work

We found that *modB* undergoes rapid phase variation in *N. gonorrhoeae*, with transitions between the ON and OFF states occurring multiple times within a single outbreak. In the absence of mutations typically associated with decreased azithromycin susceptibility, *modB* ON exhibited a clear association with elevated MICs. Transcriptomic analyses will be undertaken to define the regulon controlled by *modB* methylation, and functional genetics should be performed to directly assess the importance of the gene and its regulon in determining drug susceptibility. We also note that the ON/OFF status of phase variable genes is invisible to kmer-based genome-wide association studies (GWAS), as most repeat tracts are far longer than typical kmer sizes. Including phase variants in GWAS approaches might significantly improve the sensitivity of these approaches.

Methods

Illumina-only and Illumina+nanopore hybrid assemblies were generated as described previously (7). For analyses of base modifications, nanopore reads were demultiplexed using Deepbinner (8). The methylation load at specific target motifs was quantified from the raw nanopore fast5 reads using Tombo (4).

A ST-9363 whole-genome alignment was generated using parsnp (9), employing a closed hybrid assembly of the ST-9363 isolate 567616 as reference. A whole-genome alignment retaining all reference positions was generated using an in-house script (https://github.com/krepsen/parsnp2fasta). A recombination-corrected maximum likelihood phylogeny was generated using Gubbins (10). The Gubbins output was loaded into BactDating (11). Root-to-tip regression performed in BactDating revealed a sufficient temporal signal (Fig. S2). $4x10^7$ markov chain monte carlo steps were performed to generate a time-resolved tree (Figs. S3 and S4). The resulting evolutionary rate was 5.67 (95% CI: 3.81-8.28) mutations/genome/year which is similar to earlier studies of *N. gonorrhoeae (7, 12)*.

A neighbor-joining phylogeny of all available Norwegian *N. gonorrhoea* genomes for which *modB* tract lengths could be determined, was generated using PopPunk and RapidNJ (13, 14). The tree was visualized and edited in GrapeTree (15).

To determine the length of phase variable tracts, assemblies were aligned against the *modA*, *modB* and *hsdS* genes of FA1090 using BLAST (16).

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Figures

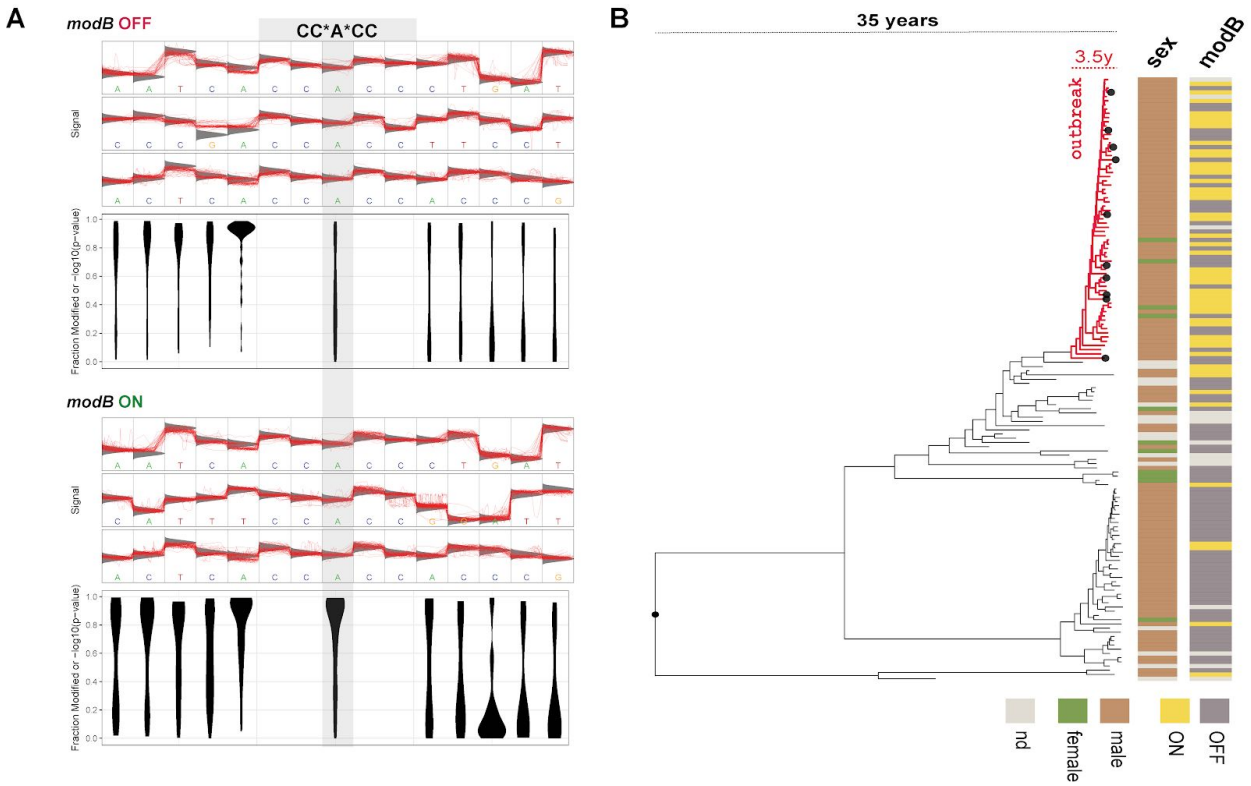


Fig. 1. Phase variation of *modB* during the course of an outbreak affects methylation status of CC*A*CC motif. A) Methylation-load on CC*A*CC target sites in isolates stratified by inferred *modB* status (ON/OFF). A “top-heavy” dumbell indicates that a high fraction of nucleotides at the target nucleotide is methylated. B) Time-resolved ST-9363 phylogeny constructed from genome-wide SNPs. The sex of individual patients are annotated together with *modB* ON/OFF status. A dense outbreak representing less than 3.5 years of evolution is highlighted in red. Isolates selected for methylome characterization are marked with black dots. As the assemblies were based on Illumina short reads, the repeat tract of the *modB* gene was split between contigs in some assemblies, and the ON/OFF status could thus not be assessed (light grey shading).

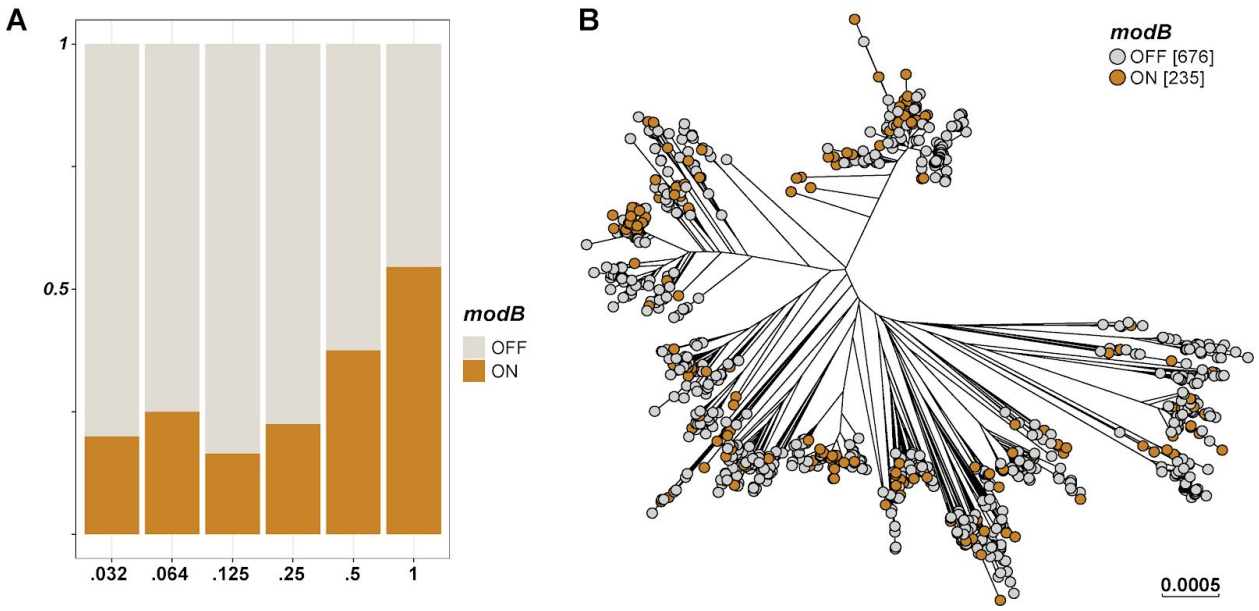


Figure 2. Configuration of *modB* as a function of azithromycin MIC. A. Within each MIC group, the fraction of isolates carrying *modB* in ON and OFF configuration is shown. B Phylogenetic tree of all isolates with *modB* status mapped to the tips. Orange = ON, Black = OFF.

References

1. Sánchez-Busó L, Golparian D, Parkhill J, Unemo M, Harris SR. 2019. Genetic variation regulates the activation and specificity of Restriction-Modification systems in Neisseria gonorrhoeae. Sci Rep 9:14685.

2. Srikhanta YN, Dowideit SJ, Edwards JL, Falsetta ML, Wu H-J, Harrison OB, Fox KL, Seib KL, Maguire TL, Wang AH-J, Maiden MC, Grimmond SM, Apicella MA, Jennings MP. 2009. Phasevarions mediate random switching of gene expression in pathogenic Neisseria. PLoS Pathog 5:e1000400.

3. Jen FE-C, -C. Jen FE, Seib KL, Jennings MP. 2014. Phasevarions Mediate Epigenetic Regulation of Antimicrobial Susceptibility in Neisseria meningitidis. Antimicrobial Agents and Chemotherapy.

4. Stoiber MH, Quick J, Egan R, Lee JE, Celniker SE. 2016. De novo identification of DNA modifications enabled by genome-guided nanopore signal processing. BioRxiv.

5. Wick RR, Judd LM, Gorrie CL, Holt KE. 2017. Unicycler: Resolving bacterial genome assemblies from short and long sequencing reads. PLoS Comput Biol 13:e1005595.

6. Grad YH, Harris SR, Kirkcaldy RD, Green AG, Marks DS, Bentley SD, Trees D, Lipsitch M. 2016. Genomic Epidemiology of Gonococcal Resistance to Extended-Spectrum Cephalosporins, Macrolides, and Fluoroquinolones in the United States, 2000-2013. J Infect Dis 214:1579–1587.

7. Osnes MN, Didelot X, de Korne-Elenbaas J, Alfsnes K, Brynildsrud OB, Syversen G, Nilsen Ø, de Blasio BF, Caugant DA, Eldholm V. The sudden emergence of a Neisseria gonorrhoeae strain with reduced susceptibility to extended-spectrum cephalosporins, Norway.

8. Wick RR, Judd LM, Holt KE. 2018. Deepbinner: Demultiplexing barcoded Oxford Nanopore reads with deep convolutional neural networks. PLoS Comput Biol 14:e1006583.

9. Treangen TJ, Ondov BD, Koren S, Phillippy AM. 2014. The Harvest suite for rapid core-genome alignment and visualization of thousands of intraspecific microbial genomes. Genome Biol 15:524.

10. Croucher NJ, Page AJ, Connor TR, Delaney AJ, Keane JA, Bentley SD, Parkhill J, Harris SR. 2015. Rapid phylogenetic analysis of large samples of recombinant bacterial whole genome sequences using Gubbins. Nucleic Acids Res 43:e15.

11. Didelot X, Croucher NJ, Bentley SD, Harris SR, Wilson DJ. 2018. Bayesian inference of ancestral dates on bacterial phylogenetic trees. Nucleic Acids Res 46:e134.

12. Didelot X, Dordel J, Whittles LK, Collins C, Bilek N, Bishop CJ, White PJ, Aanensen DM, Parkhill J, Bentley SD, Spratt BG, Harris SR. 2016. Genomic Analysis and Comparison of

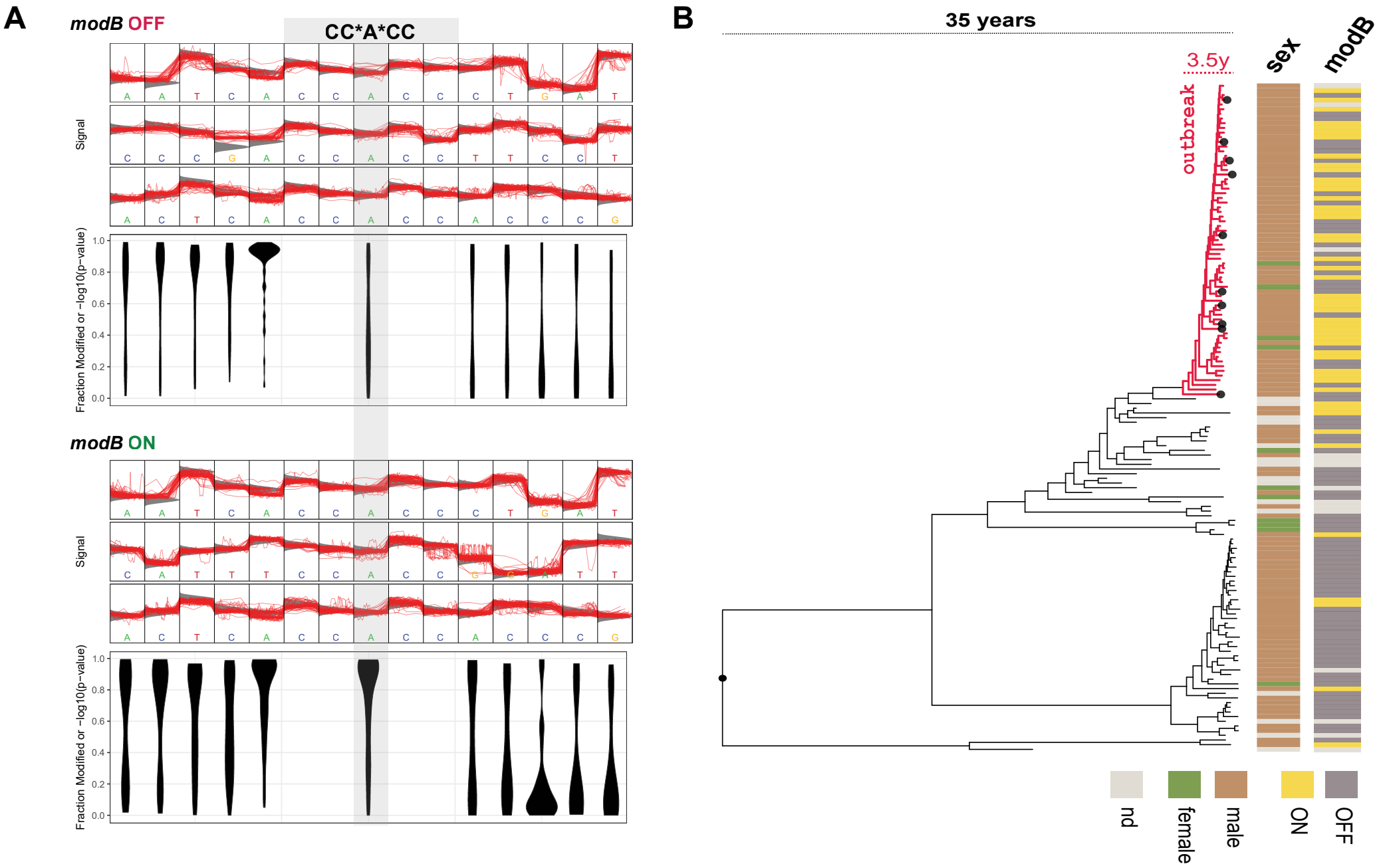
Two Gonorrhea Outbreaks. MBio 7.

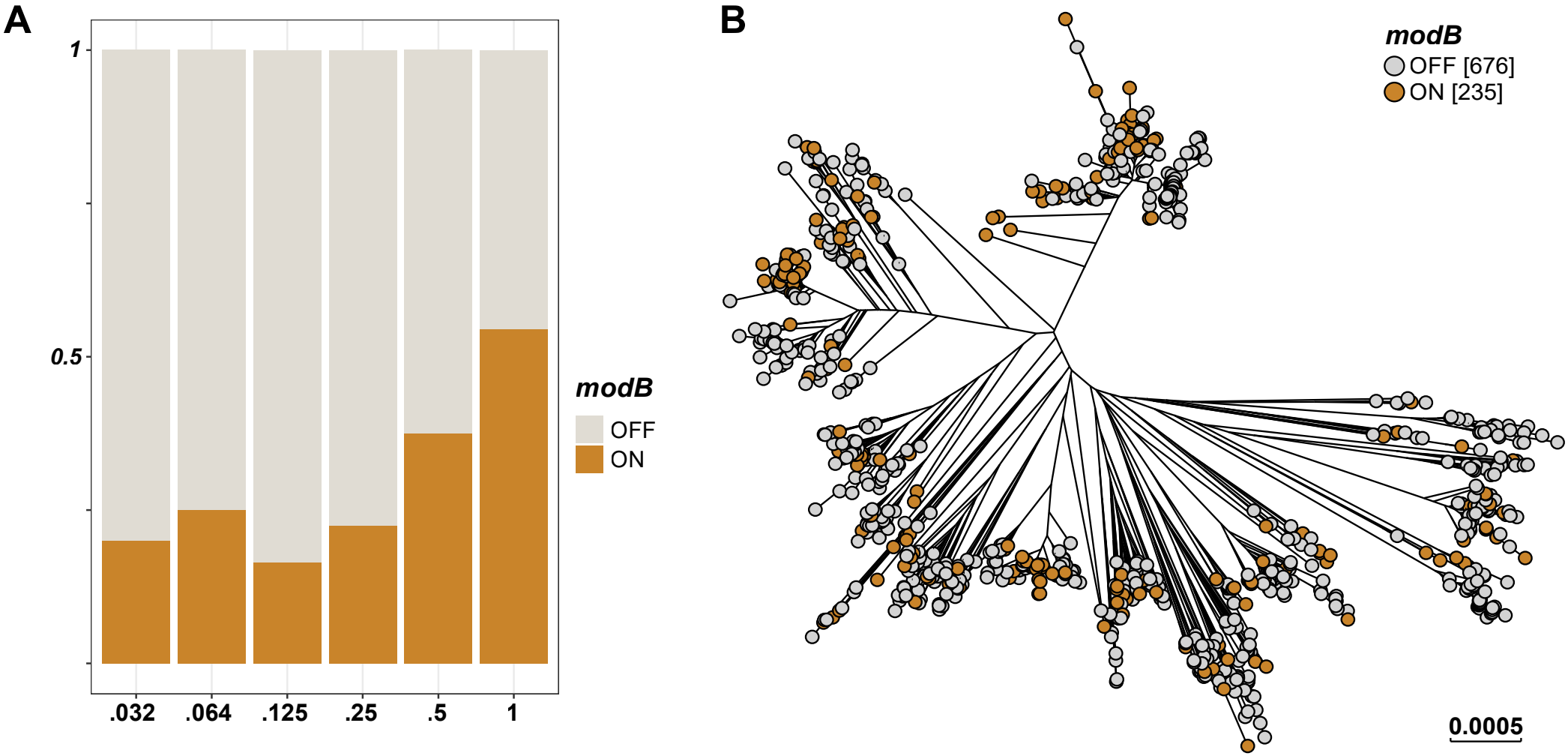
13. Lees JA, Harris SR, Tonkin-Hill G, Gladstone RA, Lo SW, Weiser JN, Corander J, Bentley SD, Croucher NJ. 2019. Fast and flexible bacterial genomic epidemiology with PopPUNK. Genome Res 29:304–316.

14. Simonsen M, Pedersen CNS. 2011. Rapid computation of distance estimators from nucleotide and amino acid alignments, p. 89–93. *In* Proceedings of the 2011 ACM Symposium on Applied Computing. Association for Computing Machinery, New York, NY, USA.

15. Zhou Z, Alikhan N-F, Sergeant MJ, Luhmann N, Vaz C, Francisco AP, Carriço JA, Achtman M. 2018. GrapeTree: visualization of core genomic relationships among 100,000 bacterial pathogens. Genome Res 28:1395–1404.

16. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. J Mol Biol 215:403–410.


A
modB OFF
CC*A*CC
Signal
Fraction Modified or −log10(p−value)
modB ON
B
35 years
3.5y
outbreak
Sex
modB
nd
female
male
ON
OFF


A
1
0.5
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.064
.125
.25
.5
1
modB
OFF
ON
B
modB
OFF [676]
ON [235]
0.0005