ECCMID 2024 | Poster #P1694

High-level plazomicin exposure selects for mutations in *fusA*, *ratA*, and a novel two-component system in *Morganella morganii*

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Introduction

- Plazomicin, a semi-synthetic aminoglycoside, has broad activity against Enterobacterales carrying diverse aminoglycoside resistance (AMG^R) mechanisms.
- Plazomicin nonsusceptibility is associated with target site modification via transferrable 16S rRNA methyltransferases.
- From 2018–2022, the SENTRY Antimicrobial Surveillance Program identified only 12 isolates with plazomicin MICs ≥128 mg/L out of >9,600 clinical Enterobacterales screened; 11/12 possessed ≥1 16S rRNA modification enzyme.
- A single *Morganella morganii* isolate (plazomicin MIC, 128 mg/L) obtained in 2022 contained no identifiable AMG^R genes and was investigated for intrinsic resistance mechanism.

Materials and Methods

- Nine *M. morganii* isolates from the SENTRY Antimicrobial Surveillance Program were selected as parental strains for single-step mutagenesis under plazomicin selection.
- Baseline agar dilution MICs were determined according to CLSI on Mueller-Hinton agar and using a modified method on lysogeny agar (LA).
- Single-step selection with plazomicin was performed on LA using doubling dilutions of 2- to 16-fold the baseline value.
- Resistant colonies were selected after 24 h, passaged non-selectively twice on tryptic soy agar with 5% sheep's blood, and susceptibility tested against plazomicin and comparator aminoglycosides (gentamycin, tobramycin, and amikacin) using broth microdilution (BMD) according to CLSI.
- Parental strains were sequenced using short-read Illumina and long-read Oxford nanopore methods to create whole genome reference sequences.
- Daughter isolates displaying stable ≥4-fold increases in plazomicin MIC values were Illumina-sequenced and single-nucleotide polymorphism (SNP) and insertion/deletion (indel) analysis was performed relative to the respective parent strain.

Results

- Twenty daughter strains with 4- to 32-fold increases in plazomicin MIC values were further characterized for mechanisms underlying development of resistance.
- Twelve daughters acquired mutations in the response regulator (2/12) or sensor kinase (10/12) of a putative OmpR/EnvZ-like two component system, RssA/RssB.
  - Isolates with mutations in the putative response regulator RssB displayed ≥32-fold increases in plazomicin MIC values while other aminoglycoside MIC values increased 2- to 8-fold.
  - Mutations in the putative sensor kinase RssA were identified in isolates displaying plazomicin MIC increases of 16- to >32-fold; MIC values for other aminoglycosides displayed 2- to 16-fold increases.
- Eight isolates possessed mutations outside of RssA/RssB.
  - Two daughter isolates possessed mutations in a putative 50S ribosomal rRNA-targeting anti-association toxin, RatA, which displayed MIC value increases of 4- to 8-fold for plazomicin and 4- to 16-fold for other aminoglycosides.
  - FusA (elongation factor G) mutations were identified in two daughter isolates, both of which increased plazomicin MIC values 8-fold; similar fold increases in MIC values were observed for other aminoglycosides.
  - Single daughter isolates each bore alterations in UbiI, HemD, NikA2, and MlaA, which produced 4- to 8-fold higher MIC values for plazomicin, relative to their respective parent strain.
- Relative to the nine *M. morganii* isolates used as parental strains, MM2022 possessed T195P and D384E alterations in RssA.
  - Parental isolate MM3 (plazomicin MIC, 1 mg/L) also possessed alterations in RssA (A27T and D384E) and MM8 (MIC, 1 mg/L) bore a nonsense mutation in RssB; neither parental isolate produced daughters with mutations in RssA/RssB.
  - An alteration in RatA (E43G) was also identified in MM2022; parental isolates MM1 (MIC 4 mg/L) and MM2 (MIC 2 mg/L) maintained D43 and G43 residues, respectively, in contrast to E43 in all other parental strains.

Conclusions

- *M. morganii* isolates exposed to plazomicin concentrations above the MIC produced daughter strains with mutations in known (FusA) and novel (RssA/RssB, RatA) targets influencing susceptibility to aminoglycoside agents.
- Many daughter isolates bore mutations in the putative sensor kinase component, RssA, of an uncharacterized two-component regulatory system, RssA/RssB.
- Larger fold increases in plazomicin MIC values were observed in daughter strains possessing mutations in RssA/RssB (16- to >32-fold) relative to those daughter isolates bearing non-RssA/RssB mutations (4- to 8-fold), although MIC increases for other aminoglycoside agents were lower in these altered-RssA/RssB strains (2- to 16-fold).
- Parental isolates with noncanonical RssA or RssB sequences produced daughters with alterations in targets other than RssA/RssB.
- A single *M. morganii* isolate collected during the 2022 SENTRY Antimicrobial Surveillance Program possessed altered RssA and RatA proteins, which may have contributed to elevated plazomicin MIC values in the absence of known effectors (e.g., 16S rRNA methyltransferases).
- Future work will address the roles of those proteins identified in this study in controlling susceptibility to different aminoglycoside agents, the distribution of those mechanisms across different bacterial species groups, and their prevalence in clinical isolates.

Table 1. Characterization of *M. morganii* strains with reduced susceptibility to plazomicin and other aminoglycosides following exposure to plazomicin

Strain	Selection (X MIC[a])	MIC mg/L (Fold Change[b])				SNP Analysis[c]
		PLZ	GEN	TOB	AMK	
MM2022[d]	NA	128	4	2	16	RssA T195P/D384E; putative TCS-SK with RssB RatA E43G; putative 50S-binding, ribosomal anti-association toxin
MM1[e]	NA	4	1	0.5	2	
MM1_D1	8X	64 (16)	4 (4)	2 (4)	8 (4)	RssA F7L; putative TCS-SK with RssB
MM1_D2	8X	>128 (>32)	16 (16)	2 (4)	16 (8)	RssA R251H; putative TCS-SK with RssB
MM2[e]	NA	2	0.5	0.5	4	
MM2_D2	2X	64 (32)	4 (8)	1 (2)	8 (2)	RssB L22P; putative TCS-RR with RssA
MM3[f, g]	NA	1	0.5	0.5	2	
MM3_D1	8X	8 (8)	4 (8)	8 (16)	32 (16)	MlaA A230fs; phospholipid-binding lipoprotein
MM3_D2	8X	8 (8)	4 (8)	8 (16)	16 (8)	FusA G117V; elongation factor G
MM3_D3	8X	8 (8)	4 (8)	8 (16)	32 (16)	NikA2 L93M; putative nickel/peptide ABC transporter SBP
MM4	NA	1	0.5	0.5	2	
MM4_D1	2X	32 (32)	2 (4)	1 (2)	8 (4)	RssA A323D; putative TCS-SK with RssB
MM4_D2	4X	8 (8)	4 (8)	4 (8)	32 (16)	RatA L20Q; putative 50S-binding, ribosomal anti-association toxin
MM4_D3	4X	32 (32)	2 (4)	1 (2)	8 (4)	RssA A323D; putative TCS-SK with RssB
MM5	NA	2	0.5	0.5	2	
MM5_D1	8X	64 (32)	4 (8)	2 (4)	16 (8)	RssA E272K; putative TCS-SK with RssB
MM5_D2	8X	64 (32)	4 (8)	2 (4)	16 (8)	RssA E272K; putative TCS-SK with RssB
MM6	NA	2	0.5	0.5	2	
MM6_D1	8X	64 (32)	2 (4)	1 (2)	8 (4)	RssA R292Q; putative TCS-SK with RssB
MM6_D2	8X	32 (16)	1 (2)	1 (2)	8 (4)	RssA V232M; putative TCS-SK with RssB
MM6_D3	16X	8 (4)	2 (4)	4 (8)	16 (8)	RatA C34Y; putative 50S-binding, ribosomal anti-association toxin
MM7[f]	NA	4	64	2	4	
MM7_D1	2X	>128 (>32)	>128 (>2)	8 (4)	16 (4)	RssA G84V; putative TCS-SK with RssB
MM7_D2	4X	>128 (>32)	>128 (>2)	16 (8)	32 (8)	RssB G19R; putative TCS-RR with RssA
MM7_D3	4X	>128 (>32)	>128 (>2)	8 (4)	8 (2)	RssA Q293K; putative TCS-SK with RssB
MM8[g]	NA	1	0.5	0.5	2	
MM8_D1	4X	8 (8)	8 (16)	8 (16)	16 (8)	FusA P613L; elongation factor G
MM9	NA	2	1	0.5	4	
MM9_D1	2X	8 (4)	4 (4)	8 (16)	32 (8)	UbiI A334fs; ubiquinone biosynthesis, cellular respiration
MM9_D2	2X	16 (8)	8 (8)	4 (8)	32 (8)	HemD H114P; heme biosynthesis, cellular respiration

Abbreviations: PLZ, plazomicin; GEN, gentamycin; TOB, tobramycin; AMK, amikacin; MIC, minimum inhibitory concentration; SNP, single nucleotide polymorphism; SK, sensor kinase; RR, response regulator; fs, frameshift
[a] Selection MIC based on agar dilution MIC of the parent strain.
[b] Fold change MIC based on BMD MIC of the parent strain.
[c] SNP analysis results for each reported gene are based on the annotation of *M. morganii* KT.
[d] Obtained from Sentry Antimicrobial Surveillance Program 2022.
[e] MM1 and MM2 maintained RatA D43 and G43 residues, respectively, compared to a consensus E43 among other parent strains.
[f] MM3 and MM7 possessed acquired aminoglycoside profiles of *ant(3``)-Ia/aph(3`)-Ia/aph(6)-Ia/aph(6)-Id* and *aac(3)-IId/aadA5/aph(3`)-Ia*, respectively.
[g] MM3 possessed alterations in RssA (T27/E384) compared to a consensus A27 and D384 other parental strains; MM8 possessed a truncated RssB (E21Stop).

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