1 Antimicrobial resistance identification using ARIBA

1.1 Introduction

ARIBA is a tool that identifies antibiotic resistance genes. This tutorial will walk you through the analysis of the *Neisseria gonorrhoeae* data set used in the ARIBA paper:

> **ARIBA: rapid antimicrobial resistance genotyping directly from sequencing reads**
> Hunt M, Mather AE, Sánchez-Busó L, Page AJ, Parkhill J , Keane JA, Harris SR.
> *Microbial Genomics 2017. doi: 110.1099/mgen.0.000131*
> PMID: 29177089

1.2 Learning outcomes

By the end of this tutorial you can expect to be able to:

- Download and prepare the standard AMR databases for use with ARIBA
- Prepare your own database for use with ARIBA
- Perform QC on input data and understand why QC is important
- Run ARIBA on several samples to identify antibiotic resistance
- Understand the different flags produced by ARIBA
- Summarise ARIBA results for several samples
- Query the AMR results produced by ARIBA
- Use Phandango to visualise ARIBA results

1.3 Tutorial sections

This tutorial comprises the following sections:

1. How to use custom reference data with ARIBA
2. Run ARIBA using the custom reference data
3. View summarized results using Phandango
4. Investigate MIC data in relation to variants in the samples

1.4 Authors

This tutorial was created by Martin Hunt.

1.5 Running the commands from this tutorial

You can run the commands in this tutorial either directly from the Jupyter notebook (if using Jupyter), or by typing the commands in your terminal window.

1.5.1 Running commands on Jupyter

If you are using Jupyter, command cells (like the one below) can be run by selecting the cell and clicking *Cell -> Run* from the menu above or using *ctrl Enter* to run the command. Let's give this a try by printing our working directory using the *pwd* command and listing the files within it. Run the commands in the two cells below.

```
pwd
```

```
ls -l
```

1.5.2 Running commands in the terminal

You can also follow this tutorial by typing all the commands you see into a terminal window. This is similar to the "Command Prompt" window on MS Windows systems, which allows the user to type DOS commands to manage files.

To get started, select the cell below with the mouse and then either press control and enter or choose Cell -> Run in the menu at the top of the page.

```
echo cd $PWD
```

Now open a new terminal on your computer and type the command that was output by the previous cell followed by the enter key. The command will look similar to this:

```
cd /home/manager/pathogen-informatics-training/Notebooks/ARIBA/
```

Now you can follow the instructions in the tutorial from here.

1.6 Let's get started!

This tutorial assumes that you have ARIBA installed on your computer. For download and installation instructions, please see the ARIBA GitHub-page.

To check that you have installed the software correctly, you can run the following command:

```
ariba -help
```

This should return the following help message:

```
usage: ariba <command> <options>

ARIBA: Antibiotic Resistance Identification By Assembly

optional arguments:
  -h, --help      show this help message and exit

Available commands:

    aln2meta      Converts multi-aln fasta and SNPs to metadata
    expandflag    Expands flag column of report file
    flag          Translate the meaning of a flag
    getref        Download reference data
    micplot       Make violin/dot plots using MIC data
    prepareref    Prepare reference data for input to "run"
    pubmlstget    Download species from PubMLST and make db
    pubmlstspecies
                  Get list of available species from PubMLST
    refquery      Get cluster or sequence info from prepareref output
    run           Run the local assembly pipeline
    summary       Summarise multiple reports made by "run"
    test          Run small built-in test dataset
    version       Get versions and exit
```

To get started with the tutorial, head to the first section: How to use custom reference data with ARIBA

2 Make custom ARIBA database

For the *N. gonorrhoeae* data of the manuscript, we were interested in particular sequences and SNPs. This means using custom reference data as opposed to one of the public reference sets (the use of which is described in the ARIBA wiki page). If you just want to use a public database, then use the command "ariba getref" instead.

We are interested in several reference sequences, some of which are coding seqeunces and others are not, and particular variants in those sequences. The idea is to generate input files to ARIBA that contain all the sequences and variants of interest, using the ARIBA function aln2meta.

First, let's change to the directory with the reference data.

```
cd data/Ref/
```

2.1 Using one reference sequence

We start by describing *folP*, but the method is (nearly) the same for all other sequences.

There are several alleles of *folP* and we want to include them all the ARIBA database. We have a SNP of interest R228S in the sequence in the allele called "folP.WHO_F_01537c", which confers resistance to sulphonamides. When we run ARIBA, a particular sample may have a different allele from folP.WHO_F_01537c, but we would still like to know whether it has the SNP R228S. However, the position may not be 228 because of insertion or deletions. So we use a multiple alignment of all the reference alleles, and just supply the SNP to ARIBA in one of the alleles, in this case folP.WHO_F_01537c.

The "aln2meta" function of ARIBA needs two input files: a multiple alignment file of the alleles, and a tab-delimited file of the SNPs of interest. In this case, the SNPs file simply contains one line:

```
cat aln2meta_input/folP_in.tsv
```

There are four columns:

1. Sequence name
2. SNP (an amino acid change at position 228, where R is the wild type and S is the variant)
3. A "group name" for this SNP. This is optional and a dot "." means no group name. Putting SNPs into the same group allows ARIBA to report them together later on.
4. A description of the SNP. This will appear in ARIBA's output files to save looking up the reason the SNP is of interest.

This file is used together with the mulitple alignment file to generate input files to ARIBA when making the database. This is the command to run:

```
ariba aln2meta -variant_only aln2meta_input/folP.aln \
          aln2meta_input/folP_in.tsv coding aln2meta_output/folP
```

A few things to note about the above command:

1. The option `--variant_only` was used, which affects how ARIBA reports later on when summarizing across all samples. We are only interested in this gene being present if it has a variant that causes resistance.
2. `aln2meta_input/folP.aln` is the name of the multiple alignment file.
3. The sequence is "coding", which makes ARIBA treat it as such, in particular it will interpret the variant R228S as an amino acid change at position 228 in the translated amino acid sequence. The input sequence is still in nucleotides, not amino acids.
4. The command output three files, which can be used as input to the command `ariba prepareref` (see later).


```
ls aln2meta_output/folP*
```

Although we have many more reference sequences of interest to deal with for the complete analysis, for illustrative purposes here we can use the three files `aln2meta_output/folP*` to make an ARIBA reference database:

```
ariba prepareref -f aln2meta_output/folP.fa \
          -m aln2meta_output/folP.tsv -cdhit_clusters \
          aln2meta_output/folP.cluster test.aribadb
```

This made an ARIBA database of just those sequences and the SNP R228S in a new directory called `test.aribadb`. Let's check that it was made:

```
ls test.aribadb
```

You do not need to worry about the contents of the new directory `test.aribadb`, just know that it can be used as input to run ARIBA. However, this was for just one of the many reference sequences of interest, so we will delete it.

```
rm -r test.aribadb
```

2.2 Using all reference sequences

We need to deal with each of the reference sequences in turn by running `ariba aln2meta` on each, like in the above example with *folP*. The only difference is that some of them are non-coding sequences, which means that the command must have `noncoding` instead of `coding`. For example:

```
ariba aln2meta -variant_only aln2meta_input/16S.aln \
          aln2meta_input/16S_in.tsv noncoding aln2meta_output/16S
```

There are 10 coding sequences and two non-coding sequences. Instead of writing 12 commands, we will use two 'for loops'. First, the coding sequences:

```
for x in folP gyrA mtrR parC parE penA ponA porB1b rpoB rpsJ
       do
           ariba aln2meta -variant_only aln2meta_input/$x.aln \
           aln2meta_input/$x\_in.tsv coding aln2meta_output/$x
       done
```

And now the two non-coding sequences:

```
for x in 16S 23S
       do
           ariba aln2meta -variant_only aln2meta_input/$x.aln \
           aln2meta_input/$x\_in.tsv noncoding aln2meta_output/$x
       done
```

This has generated three files for each sequence. We will combine these to make input files for running `ariba prepareref`.

```
cat aln2meta_output/*.fa presence_absence/*.fa > Ngo_ARIBA.fa
```

```
cat aln2meta_output/*.tsv presence_absence/presence_absence.tsv \
           > Ngo_ARIBA.tsv
```

```
cat aln2meta_output/*.cluster \
           presence_absence/presence_absence.clusters \
           > Ngo_ARIBA.clusters
```

Finally, we have the three input files needed to make a single ARIBA database that has information on all the sequences and SNPs of interest. In case the directory is already there, we delete it first, then generate the database:

```
rm -rf Ngo_ARIBAdb
```

```
ariba prepareref -f Ngo_ARIBA.fa -m Ngo_ARIBA.tsv \
           -cdhit_clusters Ngo_ARIBA.clusters Ngo_ARIBAdb
```

We now have a directory `Ngo_ARIBAdb` that can be used as the reference database when running `ariba run` on each sample.

Now move on to the next part of the tutorial where we run ARIBA using the custom reference data, or return to the index.

3 Run ARIBA on all samples

This shows how to run ARIBA on a large number of samples. To save time, this notebook does not actually run any of the commands (each run of ARIBA takes a few minutes).

3.1 Reference database

First we need a reference database. You will already have one if you followed the instructions in the previous part of this tutorial (How to use custom reference data with ARIBA). Alternatively, you can use one of the public datasets that ARIBA supports. For example, to use CARD run these two commands to make an ARIBA database directory called `ariba_db`:

```
ariba getref card out.card

ariba prepareref -f out.card.fa -m out.card.tsv ariba_db
```

3.2 How to run on one sample

ARIBA needs the database directory, which we will call `Ngo_ARIBAdb` to be consistent with the previous section of the tutorial, and two sequencing reads files `reads.1.fastq.gz`, `reads.2.fastq.gz`. The command to run ARIBA is:

```
ariba run Ngo_ARIBAdb reads.1.fastq.gz reads.2.fastq.gz outdir
```

The above command will make a new directory called `outdir` that contains the results.

3.3 Run on all samples

The *N. gonorrhoeae* dataset consists of 1517 samples, and we need to run ARIBA on each sample, which can be done with a "for" loop. We assume that the reads files are named like this:

```
ERR1067813.1.fq.gz ERR1067813.2.fq.gz
ERR1067814.1.fq.gz ERR1067814.2.fq.gz
ERR1067815.1.fq.gz ERR1067815.2.fq.gz
```

Then we can run ARIBA on all samples like this (you may need to edit this command depending on how your own files are named):

```
for sample in `ls *.1.fq.gz | sed 's/\.1.fq.gz//'`
do
    ariba run Ngo_ARIBAdb $sample.1.fq.gz $sample.2.fq.gz $sample.ariba
done
```

For Sanger pathogens users only: use LSF to run all the jobs.

```
for sample in `ls *.1.fq.gz | sed 's/\.1.fq.gz//'`
do
    bsub.py 1 $sample.ariba ariba run Ngo_ARIBAdb \
    $sample.1.fq.gz $sample.2.fq.gz $sample.ariba
done
```

The output directory of each sample is called `$sample.ariba`, for example ERR1067813.ariba is the output directory for sample ERR1067813.

3.4 ARIBA output

The output files are described here.

Now go to the next part of the tutorial where we use Phandango to view the results.

You can also return to the index or revisit the previous section.

4 Viewing ARIBA results in Phandango

This section describes how to use Phandango to view a summary of ARIBA results from many samples.

The most important output file from ARIBA is the report called `report.tsv`. For this tutorial, we have all 1517 reports in the directory `data/ARIBA_reports/`.

```
ls data/ARIBA_reports | wc -l
```

See the previous section for how to generate a report file for each sample.

ARIBA has a functon called "summary" that can summarise presence/absence of sequences and/or SNPs across samples. It takes at least two ariba reports as input, and makes a CSV file that can be opened in your favourite spreadsheet program, and also makes input files for Phandango. The two Phandango files (a tree and a CSV file) can be dropped straight into the Phandango page for viewing.

The tree that ARIBA makes is based on the CSV file, which contains results of presence/absence of sequence and SNPs, and other information such as percent identity bewteen contigs and reference sequences. This means that it does not necessarily represent the real phylogeny of the samples. It is more accurate to provide a tree built from the sequencing data. For this reason, we will use a pre-computed tree file `data/tree_for_phandango.tre`.

4.1 Basic usage of ariba summary

First, let's run `ariba summary` using the default settings, except we will skip making the tree:

```
ariba summary -no_tree out data/ARIBA_reports/*.tsv
```

We can see that this made two files:

```
ls out.*
```

They are the same except for the first line, which has Phandango-specific information. ARIBA uses the filenames as sample names in the output:

```
head -n 2 out.phandango.csv
```

The first name is "data/ARIBA_reports/ERR1067709.tsv", and the rest are named similarly. This is not ideal, as it will look ugly in Phandango. Further, the names must exactly match the names in the tree file for Phandango to work (have a look in the tree file `data/tree_for_phandango.tre`). You could do a little hacking here using the Unix command `sed` on the CSV file. Instead, we can supply ARIBA with a file of filenames that also tells ariba what to call the samples in its output CSV files. Instead of "data/ARIBA_reports/ERR1067709.tsv", we would like to simply use "ERR1067709", which is cleaner and matches the tree file. It also means we can (and will) repeatedly run `ariba summary` with different options, and get output files that can be loaded straight into Phandango. This is one way to make the file with the naming information:

```
ls data/ARIBA_reports/* | awk -F/ '{print $0,$NF}' | \
          sed 's/.tsv$//' > data/filenames.fofn
```

The file is quite simple. Column 1 is the filename, and column 2 is the name we would like to use in the output.

```
head data/filenames.fofn
```

Now we can rerun summary using this input file. Note the use of the new option `--fofn`.

```
ariba summary -no_tree -fofn data/filenames.fofn \
          out data/ARIBA_reports/*.tsv
```

Check that the renaming worked:

```
head -n 2 out.phandango.csv
```

Now go to Phandango and drag and drop the files `out.phandango.csv` and `data/tree_for_phandango.tre` into the window. The result should like this:

Default Layout

This a very high-level summary of the data. For each cluster, it is simply saying whether or not each sample has a 'match'. Green means a match, and pink means not a match. For presence/absence genes, this means that the gene must simply be there to count as a match. If it is a "variant only" gene, then the gene must be there and one of the variants that we told ARIBA about earlier when generating the ARIBA database.

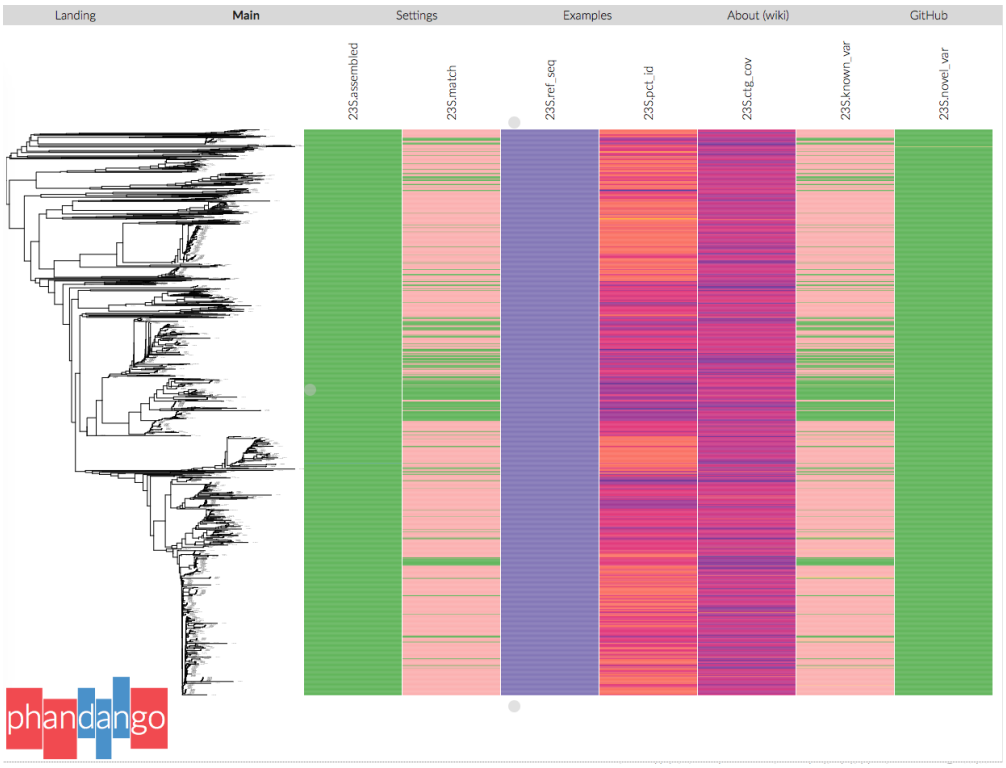
4.2 More information per cluster

In addition to a simple "yes" or "no" as to whether a sample "matches" a given cluster (as explained above), more columns can be output for each cluster. See the ARIBA summary wiki page for a full description of the options.

Adding more columns can result in a very wide plot, so we will just look at 23S from now on, using the option `--only_clusters 23S`. Adding the option `--preset all` will show all available columns for the 23S cluster:

```
ariba summary -only_clusters 23S -preset cluster_all -no_tree \
        -fofn data/filenames.fofn out data/ARIBA_reports/*.tsv
```

As before, drag and drop the files `out.phandango.csv` and `data/tree_for_phandango.tre` into the window. This time the result should look like this:



Phandango 23S cluster_all

Now there are seven columns, showing various findings from ARIBA for 23S. These columns are described in the ARIBA summary wiki page.

You can control exactly which of the seven cluster columns are output using the option `--cluster_cols` instead of `--preset`. For example, this will show just the "match" and "pct_id" columns:

```
ariba summary -only_clusters 23S -cluster_cols match,pct_id \
          -no_tree -fofn data/filenames.fofn out \
          data/ARIBA_reports/*.tsv
```

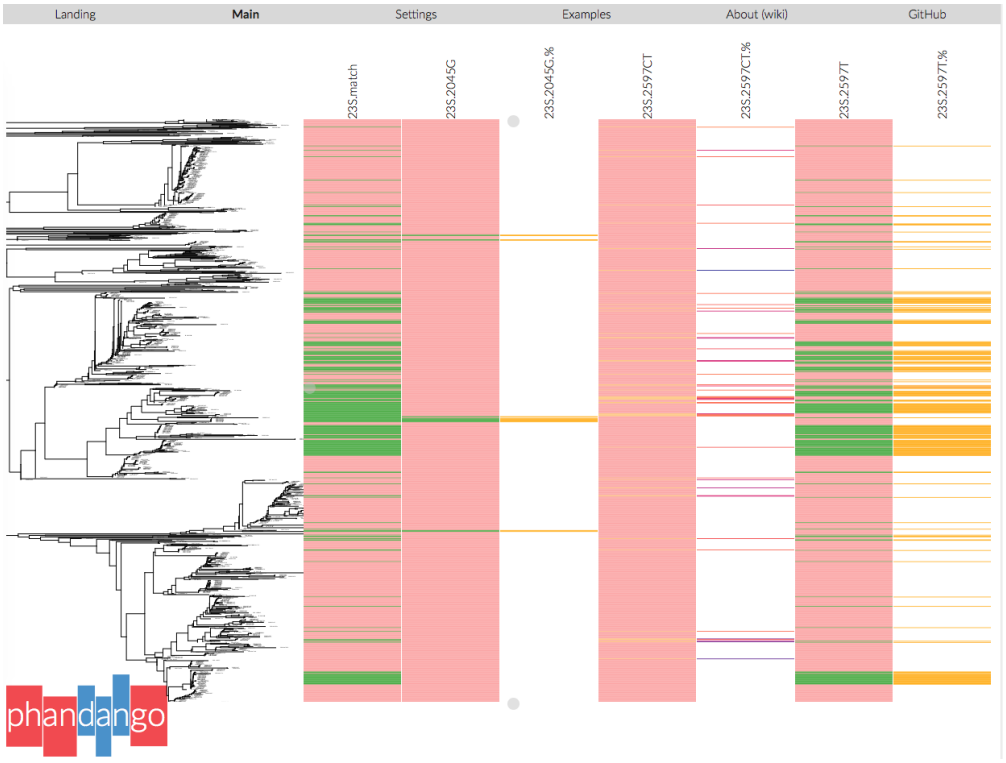
4.3 Variants

In the previous screenshot, where the option `--preset cluster_all`, there are two variant columns: "known_var" and "novel_var". Green means "yes" and pink means "no". Here, we are considering a variant to be a difference bewteen the reference sequence and the assembly contig from the reads. The known_var column indicates whether each sample has any variant that is known to ARIBA, which means it was included when the original ARIBA database was generated. The novel_var column indicates whether or not a sample has any variant that is not already known to ARIBA.

We can view the calls for all the known variants by adding the `--known_variants` option:

```
ariba summary -only_clusters 23S -known_variants -no_tree \
         -fofn data/filenames.fofn out data/ARIBA_reports/*.tsv
```

As before, drag and drop the files `out.phandango.csv` and `data/tree_for_phandango.tre` into the window. This time the result should like this:

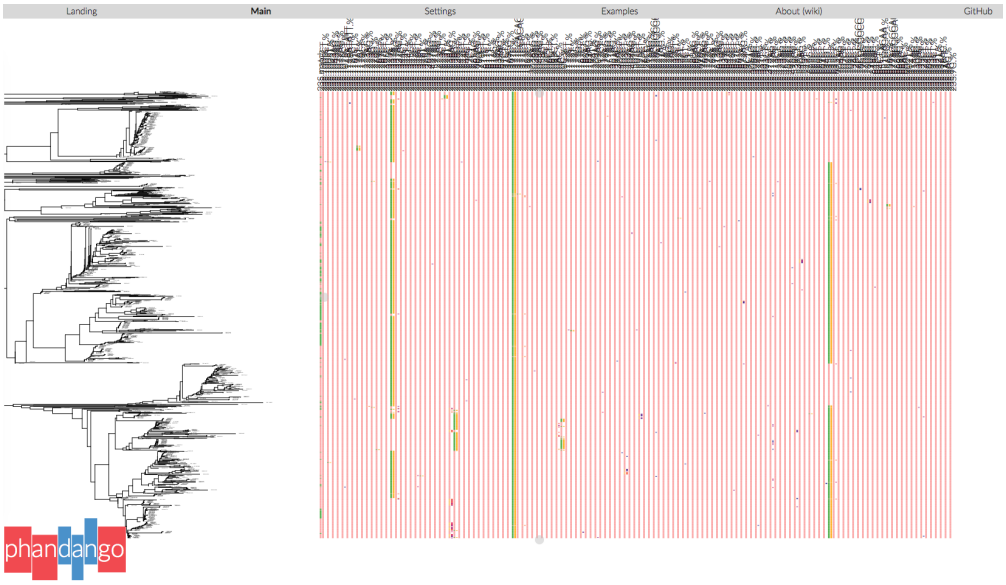


Phandango 23S known_variants

Each known SNP and whether or not it is present is shown, but you may have noticed there are three colours. Green means "yes", orange means "heterozygous", and pink means "no". *N. gonorrhoeae* has four copies of 23S. A SNP could be present in none, some, or all copies. Where it is present in 1,2, or 3 copies, it is called "heterozygous" by ARIBA. Consider the SNP 2597CT in column 4, and column 5 to the right "2597CT.%". Samples either do not have this SNP, or have heterozygous calls. The 2597CT.% shows the percent of reads that have the SNP, when mapped to the contig. Hover over the coloured blocks in Phandango to see the percentage.

```
ariba summary -only_clusters 23S -novel_variants -no_tree \
        -fofn data/filenames.fofn out data/ARIBA_reports/*.tsv
```

Now Phandango shows all variants found in any of the samples (even if the variant is unique to one sample). This results in a lot of columns!



Phandango 23S novel_variants

Now go to the next part of the tutorial where we Investigate MIC data in relation to variants in the samples.

You can also return to the index or revisit the previous section.

5 Investigating MIC data

We will use the *N. gonorrhoeae* dataset. This tutorial includes pre-computed output of running ARIBA on all the samples, and the ARIBA database that was made in the first section. Do not worry if you did not follow that part of the tutorial - we will use a pre-computed version of the database called `data/Ref/Ngo_ARIBAdb/`.

ARIBA has a function called "micplot" that generates plots showing the distribution of MICs across samples with different combinations of genotypes. To use it, a file is required of MIC data for each sample and at least one drug. It looks like this:

```
head data/mic_data.tsv
```

The first column must be named "Sample" and have names that exactly match those in ARIBA summary files used as input to micplot (we will see this shortly). The remaining columns should contain drug names and MIC scores, however, note that the first two columns contain other data that will be ignored by ARIBA. When ARIBA loads the file, it tries to convert everything in columns 2 onwards to numbers and assign a value of "NA" when this is not possible.

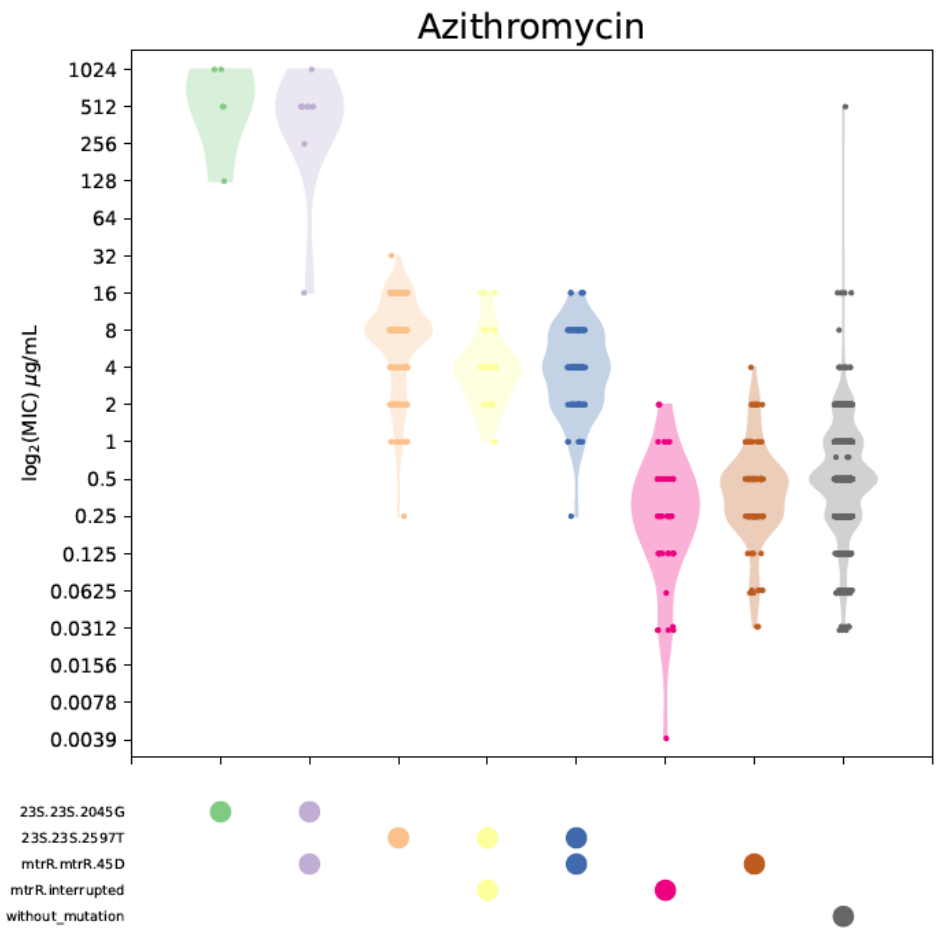
To run micplot, we need an MIC file, like the one above, and an ARIBA summary file (as described in the previous section). This generates a summary of known 23S and mtrR mutations and includes the "assembled" cluster column, so that interrupted mtrR can be identified:

```
ariba summary -row_filter n -cluster_cols assembled,known_var \
          -only_clusters 23S,mtrR -v_groups -no_tree \
          -fofn data/filenames.fofn summary.AZMknowngroups
```

Now we can run micplot using the new file `summary.AZMknowngroups.csv` and the MIC file `data/mic_data.tsv`, showing the MIC data for azithromicin compared with the different combinations of sequences and known mutations in 23S and mtrR:

```
ariba micplot data/Ref/Ngo_ARIBAdb/ -interrupted Azithromycin \
          data/mic_data.tsv summary.AZMknowngroups.csv \
          micplot.AZMknowngroups
```

This produced a pdf file `micplot.AZMknowngroups.pdf` that looks like this:



micplot AZMknowngroups

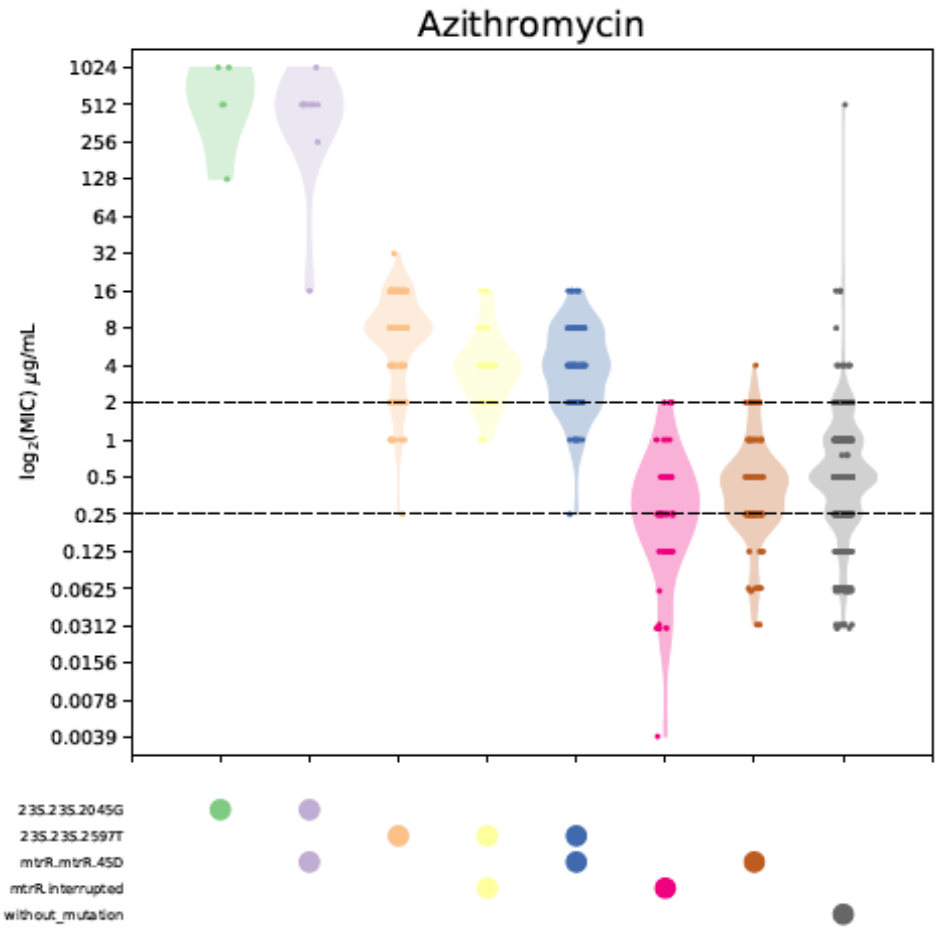
There are various options that can be changed. We will show some of them here, but try running `ariba micplot --help` to see all the options.

5.1 Horizontal lines

Horizontal lines can be added to indicate import cutoffs for MIC data, in this case 0.25 and 2, using the option `--hlines`.

```
ariba micplot data/Ref/Ngo_ARIBAdb/ -interrupted -hlines 0.25,2 \
          Azithromycin data/mic_data.tsv summary.AZMknowngroups.csv
          micplot.AZMknowngroups
```

Here is the result:



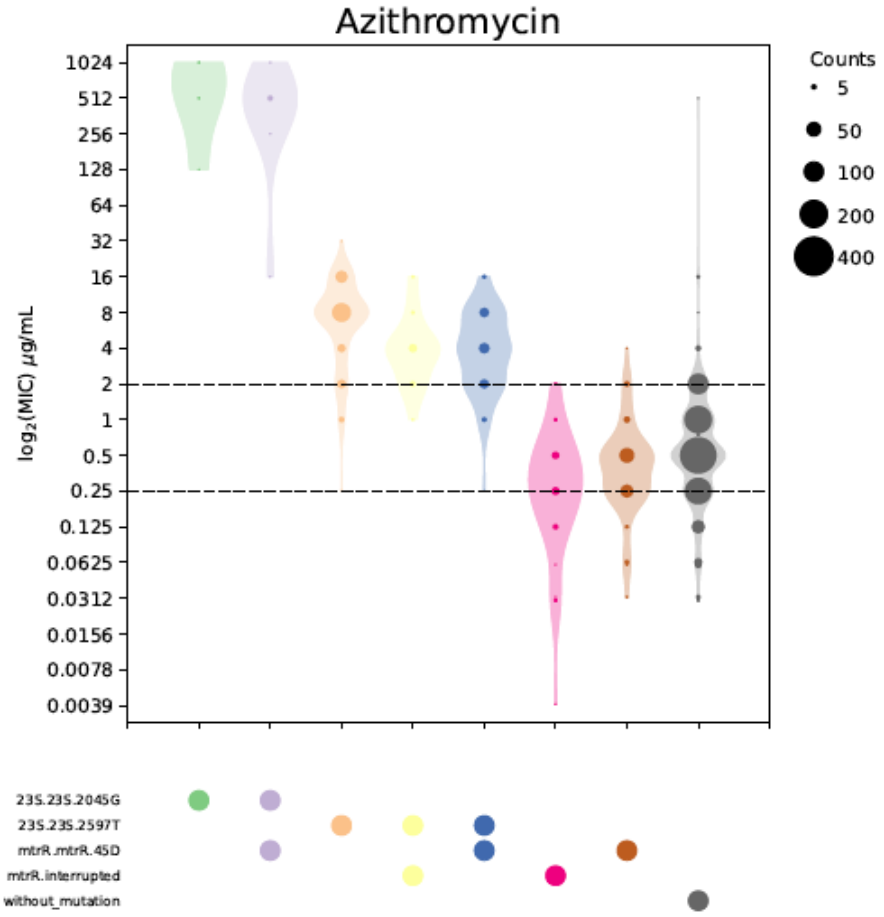
micplot hlines

5.2 Plot styles

In the plots above, there is one point per sample. It can be hard to see how many points there are, despite there being jittering applied to the horizontal position. We can change the style to group the points together and plot circles of sizes proportional to the number of samples, using the option `--point_size`. This option determines the size of the points, but when set to zero if groups the points together.

```
ariba micplot data/Ref/Ngo_ARIBAdb/ -interrupted -hlines 0.25,2 \
        -point_size 0 Azithromycin data/mic_data.tsv \
        summary.AZMknowngroups.csv micplot.AZMknowngroups
```

Here is the result:

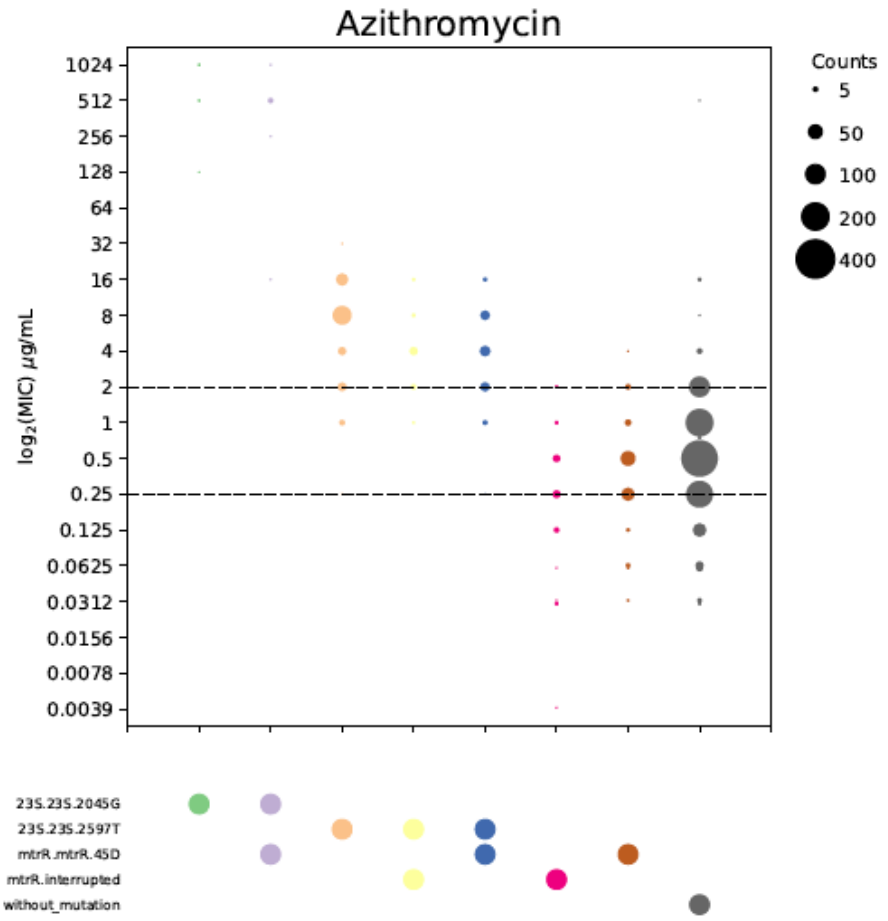


micplot point size zero

You can choose to not show the violin plots or the dots in the upper plot, using the option `--plot_types`. The default is `violin,point`, which means show both. To only show the dots:

```
ariba micplot data/Ref/Ngo_ARIBAdb/ -interrupted -hlines 0.25,2 \
        -plot_types point -point_size 0 \
        Azithromycin data/mic_data.tsv summary.AZMknowngroups.csv \
        micplot.AZMknowngroups
```

Here is the result:



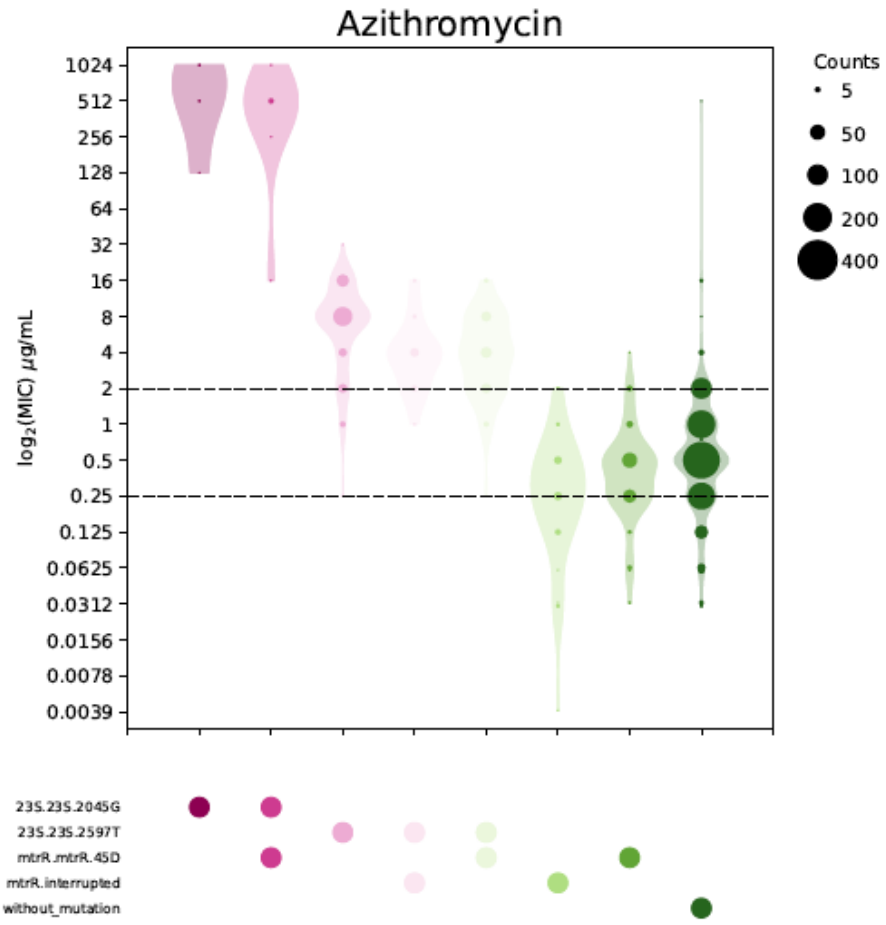
micplot dots only

5.3 Colours

There are various colour options - see the matplotlib colourmaps page for all of the available colour palettes. The default is "Accent", which has 8 colours. ARIBA will cycle through these, repeating colours if there are more than 8 columns in the plot. The palette can be changed using the option `--colourmap`.

```
ariba micplot data/Ref/Ngo_ARIBAdb/ -interrupted -hlines 0.25,2 \
        -colourmap PiYG -point_size 0 Azithromycin data/mic_data.tsv
        summary.AZMknowngroups.csv micplot.AZMknowngroups
```

Here is the result:

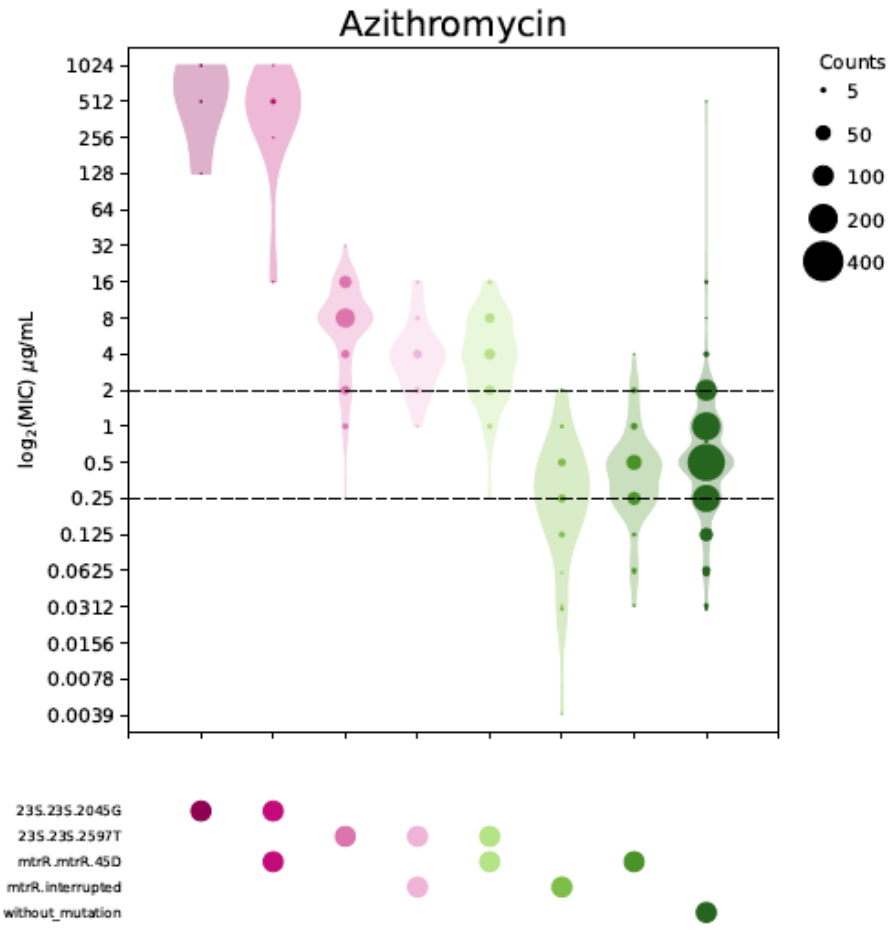


micplot PiYG

The palette PiYG is continuous, and is almost white in the middle. This is not ideal. We can skip the range in the middle, specifically 40-60%, using the option `--colour_skip`:

```
ariba micplot data/Ref/Ngo_ARIBAdb/ -interrupted -hlines 0.25,2 \
        -colourmap PiYG -colour_skip 0.35,0.65 -point_size 0 \
        Azithromycin data/mic_data.tsv summary.AZMknowngroups.csv \
        micplot.AZMknowngroups
```

Here is the new plot:

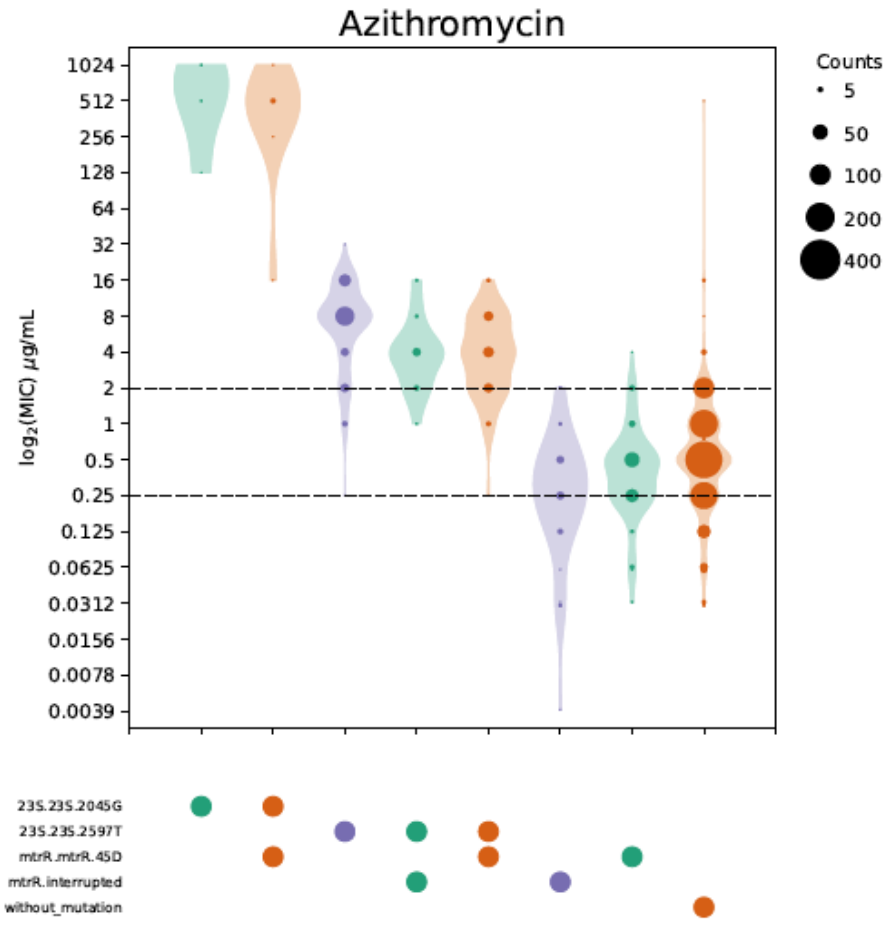


micplot colour_skip

The number of colours can be set to less than the number of columns using the option `--number_of_colours`. This makes ARIBA cycle the colours. Here is an example using the first three colours from the "Dark2" colour palette:

```
ariba micplot data/Ref/Ngo_ARIBAdb/ -interrupted -hlines 0.25,2 \
        -colourmap Dark2 -number_of_colours 3 -point_size 0 \
        Azithromycin data/mic_data.tsv summary.AZMknowngroups.csv \
        micplot.AZMknowngroups
```

And we only have three colours:

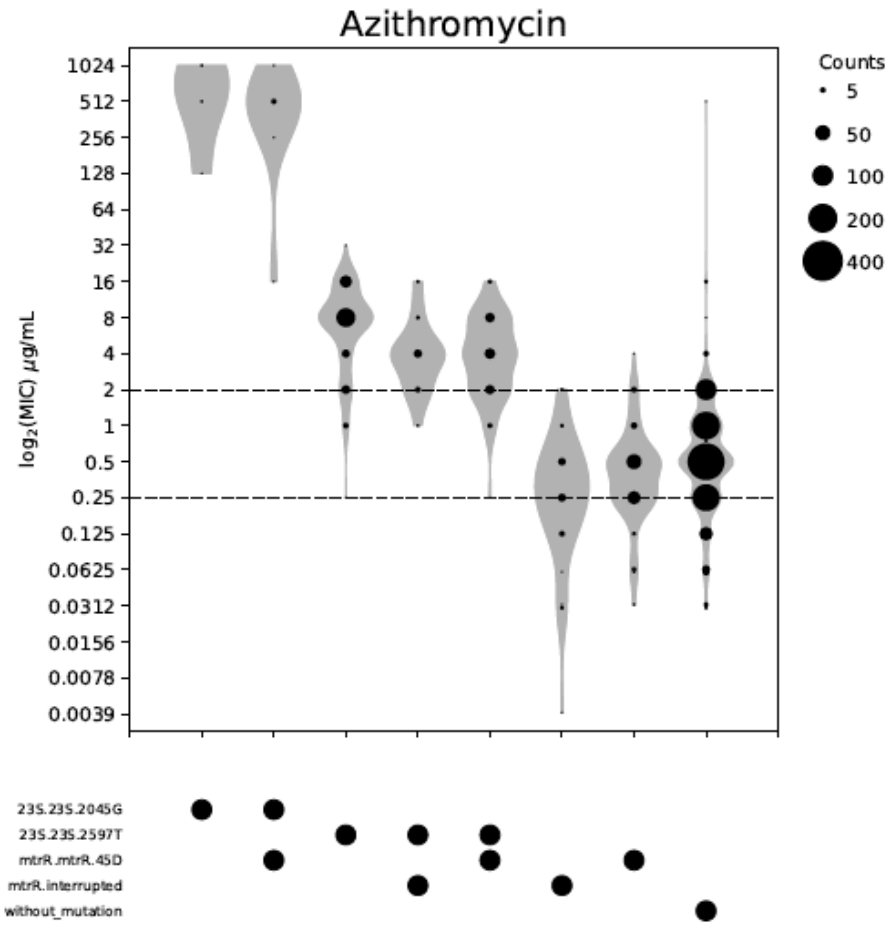


micplot 3 colours

Setting the number of colours to one results in a black and white figure.

```
ariba micplot data/Ref/Ngo_ARIBAdb/ -interrupted -hlines 0.25,2 \
        -number_of_colours 1 -point_size 0 Azithromycin \
        data/mic_data.tsv summary.AZMknowngroups.csv micplot.AZMknown
```

Here is the black and white figure:



micplot black and white

This is the end of the tutorial. You can return to the index or revisit the previous section.