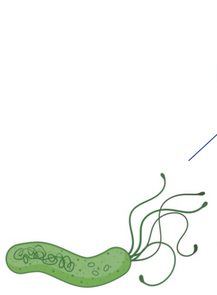
Directed evolution of tetracycline, metronidazole and levofloxacin antibiotic resistance in *Helicobacter pylori*

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Introduction



- Gram negative bacterium
  - Infects the human gastric mucosa
  - Infects ~ 50% of the population
- Can cause health issues
  - Gastritis
  - Peptic ulcers
  - Gastric cancer
- Difficult treatment
  - Culture & AST only recommended after two failed antibiotic courses in the UK
  - High mutation rate
  - Increasing antibiotic resistance[1]

- *H. pylori* eradication is impeded by increasing antimicrobial resistance (AMR).
- It is challenging to culture for downstream phenotypic antibiotic susceptibility testing (AST).
- Knowledge of genetic determinants of resistance would aid rapid genotype-based AMR prediction.

- This project aimed to:

i. Evolve resistance to antibiotics metronidazole (MZ), tetracycline (TC), and levofloxacin (LV) in clinical strains, *in vitro*.

ii. Identify the mutations involved in the progressive evolution of resistance to TC, MZ and LV.

Evolution through serial passage

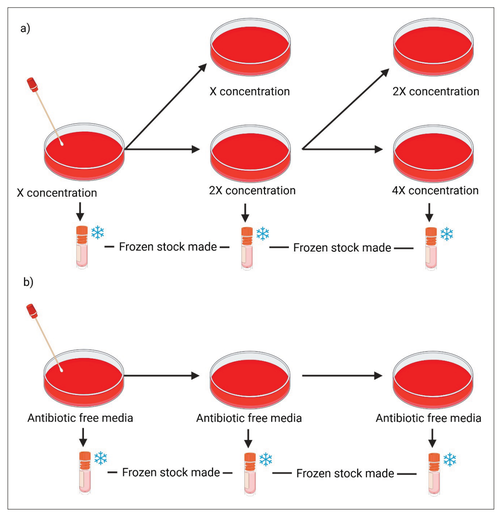


Figure 1. Overview of the experimental evolution workflow for the evolved strains (A) and control lines (B)

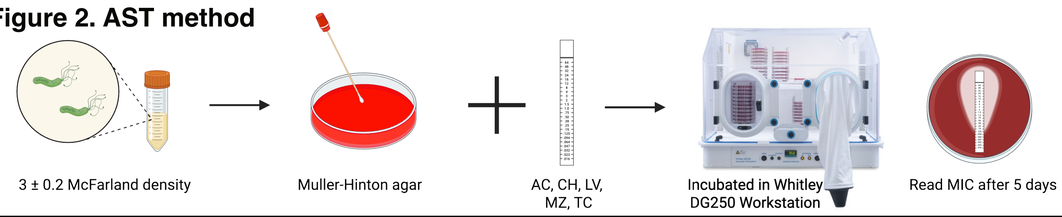
- The susceptible strain 791A was experimentally evolved using MZ.
- The susceptible strains, 295A6 & 326A25, were experimentally evolved using LV and TC.

- Overview of the experimental evolution workflow (Figure 1):
- a) Evolved lines were passaged onto antibiotic Muller-Hinton agar with a sub-MIC concentration (X). If growth was observed, the strain was passaged onto antibiotic plates with a 2X concentration and a plate with X concentration.
  e.g. 295A6 was initially passaged on a 0.016 µg/ml LV agar plate. Following growth, it was passaged onto both 0.016 µg/ml and 0.032 µg/ml LV agar plates. This process was repeated for subsequent passages.
- b) For the control lines, strains were passaged onto antibiotic free media.

AST & stability testing

- AST: The MIC was determined using E-test strips, at the endpoint of the experimental evolution, along with the corresponding control strains.
- Stability testing: The stability of the evolved resistance was tested by measuring the MIC of strains after at least three passages on antibiotic free media. The resistance was deemed to be unstable, if there was a decrease in MIC.

Figure 2. AST method



*Whitley DG250 Workstation was used for all *H. pylori* cultures

Genome sequencing & analysis

- gDNA was extracted from the evolved strains at the endpoint and midpoint of experimental evolution, along with their corresponding control strains.
- Ancestral genomes were assembled through Hybracter[3], combining Illumina short- and Nanopore long-reads, and annotated by Bakta[4].
- Illumina reads were mapped to the ancestral strain genome using breseq[2].
- Mutations identified in the control lines were removed from the analysis.
- Remaining mutations and their frequencies in the evolved lines were independently compared.
- Mutations within relevant genes, including efflux pumps, were noted along with other mutations present at high frequency.

Overview

Table 1. Showing the initial MIC of strains, their final MIC and the stability of the evolved strains to their corresponding antibiotic

Strain	Antibiotic	Initial MIC	Final MIC	x fold increase	Stable?
791A-2	MZ	0.25	>256	1024-fold	Yes
295A6	LV	0.064	>32	500-fold	Yes
	TC	0.047	0.75	15-fold	No
326A25	LV	0.38	>32	84-fold	Yes
	TC	0.032	8	250-fold	No

The EUCAST breakpoint[5] for metronidazole, levofloxacin, and tetracycline is 8 µg/ml, 1 µg/ml and 1 µg/ml, respectively.

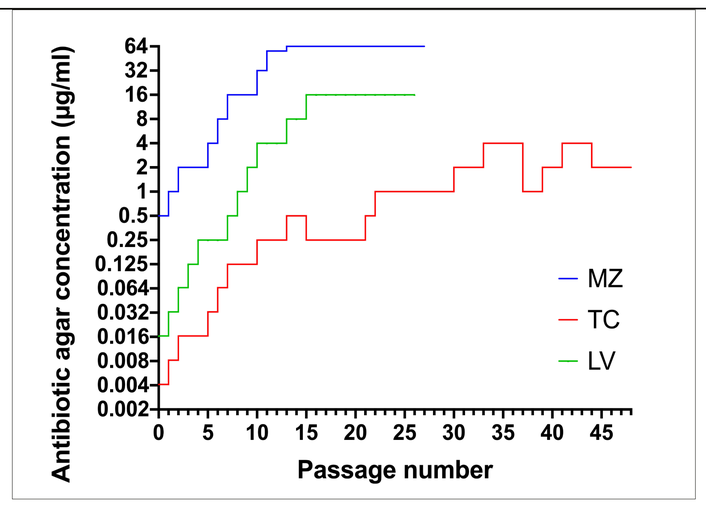


Figure 3. Showing the trajectory of representative strains experimentally evolved to MZ (791A-2), LV (326A25) and TC (326A25)

- Resistance was successfully evolved for all three antibiotics.
- The final MIC achieved is shown in Table 1. and the trajectory of the evolution shown in Figure 3.
- Strains evolved to MZ and LV were not passaged onto higher concentrations once the AST result showed no zone of clearing

- Comparing the ancestral and evolved strain genomes revealed the acquisition of single nucleotide polymorphisms (SNPs). With SNPs/mutations of significance shown in Table 2.

Table 2. Showing significant mutations in the evolved lines for the antibiotics: A) tetracycline, B) levofloxacin and C) metronidazole

A)

Strain	Gene	Nucleotide change?	Amino acid change?	Mutation in midpoint	Endpoint
295A6-TC-1	*hofH*	G→A	G251D (GGC→GAC)	Yes (fixed)	Yes (fixed)
	hefC	G→A	M377I (ATG→ATA)	No	Yes (fixed)
295A6-TC-2	*hefC*	G→A	M377I (ATG→ATA)	N/A	Yes (fixed)
	bamA	C→A	A793D	N/A	Yes
	crdB	$(ATTACAACGCTT)_{1\rightarrow 2}$	coding (1129/1239 nt)	N/A	Yes
326A25-TC-2	*hofH*	G→A	W347* (TGG→TGA)	Yes	No
		$(CATGGGGAATCCAAGCGGTGTGAT)_{1\rightarrow}$	Coding (1073/1416 nt)	Yes	No
		Δ116 bp	coding (674-789/1416 nt)	No	Yes (fixed)
	hefC	G→A	M377I	Yes (fixed)	Yes (fixed)
	bamA	G→T	M899I	No	Yes (fixed)
326A25-TC-3	*hofH*	$(TTGG)_{2\rightarrow 1}$	coding (804-807/1416 nt)	Yes	No
		Δ116 bp	coding (674-789/1416 nt)	No	Yes (fixed)
	hefC	G→A	M377I	Yes (fixed)	Yes (fixed)
	bamA	G→T	M899I	Yes	Yes (fixed)

B)

Strain	Gene	Nucleotide change?	Amino acid change?	Mutation in midpoint	Endpoint
295A6-LV-2	*gyrA*	G→A	D91N	Yes (fixed)	Yes (fixed)
		G→A	G302D	No	Yes
		G→T	E329*	No	Yes
	gyrB	G→A	S429N	No	Yes (fixed)
	hefC	C→A	A647D	No	Yes (fixed)
		C→A	I1017I	Yes	No
295A6-LV-3	*gyrA*	G→A	D91N	Yes	No
		G→T	D91Y	No	Yes (fixed)
		G→A	D155N	No	Yes
	gyrB	G→A	E463K	No	Yes (fixed)
	hefC	G→T	E58D	No	Yes (fixed)
		G→T	R545S	No	Yes (fixed)
326A25-LV-3	*gyrA*	G→A	D91N	No	Yes
		G→T	D91Y	Yes	No
	gyrB	G→A	E463K	No	Yes (fixed)
	hefC	N/A	N/A	N/A	N/A

C)

Strain	Gene	Nucleotide change?	Amino acid change?	Mutation in midpoint	Endpoint
791A-2	*rdxA*	C→T	Q65*	Yes	Yes
		A→G	R200G	Yes	Yes (fixed)
	nfnB/frxA	$(T)_{7\rightarrow 6}$	Frameshift	Yes (fixed)	Yes (fixed)
791A-3	*rdxA*	C→T	R16C	Yes	No
		C→A	P51T	Yes	No
		C→T	A67V	Yes	Yes (fixed)
		G→T	R191M	Yes	No
	nfnB/frxA	$(T)_{7\rightarrow 6}$	Frameshift	No	Yes (fixed)
		G→A	A15V	Yes	Yes

- Tetracycline:
- Unstable TC resistance evolved, with no mutations present in the 16S rRNA.
- Resistance seemed primarily driven by mutations in the efflux pump and outer membrane protein genes *hofH*, *hefC* and *bamA*.

- Levofloxacin:
- Stable LV resistance evolved, with mutations occurring in the *gyrA* and *gyrB* genes.
- Mutations at *gyrA* D91 were acquired first at the midpoint, with endpoints also containing mutations in *gyrB* which together are likely responsible for the high level of resistance (MIC >32 µg/ml).

- Metronidazole:
- Stable MZ resistance evolved, with mutations occurring in the nitroreductase *rdxA* and *frxA* genes.
- The most frequent mutation was a frameshift in *frxA*, resulting in a stop codon and truncated protein, likely responsible for the high level of resistance (MIC >256 µg/ml).

Conclusion

- Experimental evolution can provide insights into how *H. pylori* develops resistance to antibiotics.
- In the MZ-resistant strains, mutations were in the genes *frxA* and *rdxA*, with the resistance being stable.
- In the LV-resistant strains, mutations were in the genes *gyrA* and *gyrB*, with the resistance being stable.
- This is consistent with the resistance mechanisms reported by others.
- TC resistance was harder to achieve and unstable. Highlighting a potential role of the genes *hofH*, *hefC* and *bamA*, requiring further investigation.

Abbreviations

AST – Antibiotic susceptibility testing
AMR – Antimicrobial resistance
MZ - Metronidazole
TC - Tetracycline
LV - Levofloxacin
MIC - Minimum inhibitory concentration
SNP - Single nucleotide variation

Ethics statement

Ethical approval for use of *H. pylori* clinical isolates was granted by the NHS National Research Ethics Service, Nottingham Research Ethics Committee (Ref: 08/H0408/195) 27th January 2009.

Acknowledgements

Farah Mahmood was funded through a Nottingham BBSRC Doctoral Training Program studentship.
Gastric biopsy collection and gathering of the strain collection funded from NIHR Nottingham Biomedical Research Centre, Nottingham University Hospitals NHS trust and the University of Nottingham.
Attendance to this event was support by the Microbiology Society through an External Event Grant and Don Whitley Scientific through a Workstation Travel Grant.

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