Evolutionary trajectories to amoxicillin-clavulanic acid resistance in *Escherichia coli* are affected by growth media.

Alasdair T. M. Hubbard[1], Nicholas Feasey[1, 2] and Adam P. Roberts[1, *]

[1]Liverpool School of Tropical Medicine, Pembroke Place, Liverpool L3 5QA

[2]The Malawi Liverpool Wellcome Trust Clinical Research Programme, University of Malawi, the College of Medicine, Blantyre, Malawi.

*Corresponding author: adam.roberts@lstmed.ac.uk

Abstract

Antibiotic resistant bacterial isolates are routinely generated in the laboratory using sub-inhibitory concentrations of the antibiotics in a range of media to study various aspects of evolutionary biology. We wanted to determine whether growth in different media affects the selection of amoxicillin-clavulanic acid resistant *Escherichia coli*. Using a susceptible clinical isolate of *E. coli* resistance was selected for after 24-hour exposure to sub-MIC levels of amoxicillin-clavulanic acid in defined (M9), semi-defined (Iso-sensitest broth) and undefined (LB broth) media. Resistant colonies were isolated on LB agar containing 8 (MIC) or 16 μg/ml (2xMIC) amoxicillin-clavulanic acid. Fitness costs of three resistant lineages from each media-type were assessed using a competitive fitness assay and mutations in the *ampC* promoter region were identified through PCR. No resistant colonies were recovered with pre-incubation in M9, whereas Iso-sensitest and LB broth incubation resulted in growth at 8 and 16 μg/ml of amoxicillin-clavulanic acid, respectively. We observed within media-type variability in the fitness costs associated with resistance and showed that in some cases the fitness is dependent on the media in which the assay is carried out. Our data highlights the importance of media consideration when interpreting the results of evolutionary studies which will ultimately be translated into the clinic.

Introduction

In recent years, and in direct response to the global rise in antimicrobial resistant (AMR) pathogenic bacteria, there has been an increased interest in how resistance develops and becomes fixed within a bacterial population. This ranges from the effect of antimicrobials entering the environment following use in humans and animals [*1*] to understanding the evolution of antimicrobial resistance and the selective pressure from antimicrobials [*2, 3*], with a view to developing new strategies to prevent development of AMR in the future.

From an evolutionary perspective, there is a particular focus on the effect of acquisition of antimicrobial resistance has on the fitness of bacteria with a view to suppress the development of resistance [*3-5*]. Much of this work relies on the development of resistance in the presence of sub-inhibitory concentrations of single, or combinations of, antimicrobials and investigators employ a range of different media for the generation of resistance [*4, 6-8*], and the subsequent assessment of fitness related to acquired antimicrobial resistance [*9, 10*]. However, the media currently used to grow bacteria *in vitro* is not necessarily the most suitable substitute for the environment that the bacteria live in *in vivo*. Also, a recent study by Knöppel *et al*. 2017 found that mutations that confer antibiotic resistance can arise following evolution in LB broth in the absence of an antimicrobial, suggesting that media can have a direct and significant effect on bacterial evolution [*6*]. Therefore, if this work is to be truly translational, it is necessary to ensure that it *in vitro* experiments are interpreted correctly.

In this preliminary investigation, we have evaluated whether the type of media used in evolutionary studies affects the evolutionary trajectories of a clinical *Escherichia coli* isolate from Malawi [*11*] in the presence of sub-inhibitory amoxicillin-clavulanic acid. To achieve this, we have used representatives of the different commonly used media types used in evolutionary studies, M9

(defined media) [*4*], Iso-sensitest broth (semi-defined media) [*12*] and LB broth (undefined) [*6*]. This potentially has far reaching implications on the interpretation and subsequent translation of previous and future evolutionary studies of bacteria, including between study comparisons.

Materials and methods

Bacterial strains, media and antibiotics

The bacterial strain used in this study was the clinical isolate *E. coli* 10129 which was isolated from a CSF sample and susceptible to ampicillin, chloramphenicol, co-trimoxazole, gentamicin, ciprofloxacin and ceftriaxone by Musicha *et al*. 2017 [*11*]

Cultures were grown in Mueller Hinton broth (MHB), LB broth (Lennox), Iso-sensitest (Sigma, UK) or M9 (50% (v/v) M9 minimal salts (2x) (ThermoFisher Scientific, USA), 0.4% D-glucose, 4mM magnesium sulphate and 0.05 mM calcium chloride (all Sigma, UK). *E. coli* 10129 was grown on and colony forming units (CFU) counts were determined using LB agar (Lennox) (Sigma, UK).

Amoxicillin trihydrate: potassium clavulanate (4:1) (AMC) was diluted in molecular grade water (both Sigma, UK) to a stock concentration of 1 mg/ml and filter sterilised through a 0.22 µM polyethersulfone filter unit (Millipore, US).

Minimum inhibitory concentrations

Minimum inhibitory concentrations (MIC) were determined using cultures grown in MHB, unless otherwise stated, in the absence of antimicrobial selection and following the Clinical and Laboratory Standards Institute guidelines for broth microdilution MIC and determined visually.

Selection of E. coli *10129 resistant to amoxicillin-clavulanic acid*

E. coli 10129 was grown in sub-inhibitory concentrations of AMC to select for resistant isolates. Separate cultures of *E. coli* 10129 were initially grown in 10 ml LB broth, Iso-sensitest broth or M9 for 18 hours at 37°C and shaking at 200 rpm. The cultures were serially diluted 1 in 10 in phosphate buffered solution (PBS, pH 7.2) (ThermoFisher Scientific, USA) and 50 µl of the neat to 10^{-7} dilutions were plated out on to LB agar and incubated at 37°C for 18 hours to determine CFU/ml of the culture. Using the initial cultures, 10 µl was diluted in 10 ml of the respective test media containing 4 µg/ml AMC and incubated at 37°C with shaking at 200 rpm for 24 h. The cultures were serially diluted in PBS as described above and 50 µl of the neat to 10^{-7} dilutions were plated onto LB agar and 50 µl of neat to 10^{-2} dilutions were plated onto LB agar + 8 µg/ml AMC (MIC) and LB agar + 16 µg/ml AMC (2 x MIC). These plates were incubated at 37°C for 18 h. Up to 10 single colonies were selected from agar plates from three replicate resistant selection experiments which had growth at the highest concentration of AMC and immediately stored in 1 ml LB broth + 40% glycerol (Sigma, UK) and stored at -80°C to prevent any further growth or compensatory mutations. The number of colonies were counted at the dilution that grew between 15-100 colonies, except for those that only grew small number of colonies at the neat dilution.

Competitive Fitness Assay

The competitive fitness of the resistant isolates in comparison to the ancestral isolate *E. coli* 10129 was determined. Cultures of ancestor and resistant isolates were inoculated in LB broth, Iso-sensitest broth or M9 directly from the -80°C to minimise any further evolutionary events. The ancestor isolate was grown without the presence of AMC, whereas the resistant isolates were grown in the presence of the same concentration of AMC that they were initially isolated from to confirm resistance and ensure only the resistant population grew, i.e 8 or 16 µg/ml AMC. All cultures were diluted in the appropriate media to an optical density at 600nm (OD_{600}) of 0.1. A resistant and its ancestor isolate were further diluted 1/1000 and combined 1:1, again in the corresponding media in

which resistance emerged, 150 µl of which was added to a 96 well plate and incubated at 37°C for 24 hours. The initial combined culture was serially diluted in PBS and 50 µl of the neat to 10^{-7} dilutions were plated out onto both LB agar and LB agar plus either 8 or 16 µg/ml AMC and incubated at 37°C for 18 hours. Following 24 hours of growth, the combined culture was then plated out on to agar as described above. The number of colonies were counted at the dilution that grew between 15-120 colonies.

Relative fitness was calculated using the Malthusian equation [*13*]:

$$M = M_{WT}(\frac{T_{24}}{T_0})/M_m(\frac{T_{24}}{T_0})$$

$$W = 1 - M$$

Where W is the relative fitness, M is the Malthusian parameter, M_{WT} is the Malthusian parameter of the ancestor isolate, M_m is the Malthusian parameter of the resistant isolate, T_0 is the CFU/ml count of the initial culture and T_{24} is the CFU/ml count after 24 hours.

DNA extraction

Resistant isolates were grown in 10 ml LB broth containing either 8 or 16 µg/ml AMC and the ancestral isolate was grown in 10 ml LB broth only, all were incubated at 37°C with shaking at 200 rpm for 18 hours. DNA extraction was performed using the Gentra Puregene Yeast/Bact. Kit (Qiagen, Germany). Briefly, 500 µl of each culture was centrifuged at 13000 x *g* for 5 seconds, the supernatant removed, resuspended in 300 µl Cell Lysis Solution and incubated at 80°C for 5 minutes. To each sample 1.5 µl of RNase A solution (Qiagen, Germany) was added, inverted 25 times and incubated for 1 hour at 37°C followed by cooling on ice for 1 minute. Proteins in the sample were precipitated by the addition of 100 µl Protein Precipitation solution, vortexed at high speed for 20 seconds and centrifuged at 13000 x *g* for 3 minutes. The supernatant from each sample was transferred to 300 µl 2-propanol (Sigma, UK), inverted 50 times, centrifuged at 13000 x *g* for 1

minute and the supernatant discarded. The remaining pellet was washed with 70% ethanol, centrifuged at 13000 x *g* for 1 minute, and the supernatant discarded. The pellet was allowed to air dry at room temperature for 5 minutes. The DNA was dissolved in 30 µl molecular grade water (Sigma, UK) for 1 hour at 65°C and the DNA concentration determined using a NanoDrop™ One/One^{C} Microvolume UV-vis Spectrophotometer (ThermoFisher Scientific, USA).

PCR amplification of the ampC *promoter region*

A 271-bp fragment of the promoter region of *ampC* was amplified using the following primers; AB1 5'-GATCGTTCTGCCGCTGTG-3' (between nucleotides -151 and -134) and ampC2 5'GATCGTTCTGCCGCTGTG-3' (between nucleotides +120 and +101) [*14, 15*]. The PCR reaction was performed using the Q5® High Fidelity polymerase (New England Biolabs, USA) and the final reaction contained 1x Q5® reaction buffer, 200 µM dNTPs, 0.5 µM of both AB1 and ampC2 primers and 0.02 U/µl Q5® polymerase in a total volume of 25 µl. The PCR reactions were run using the following protocol: denaturation at 98°C for 30 seconds, followed by 35 cycles of denaturation at 98°C for 10 seconds, annealing at 68°C for 30 seconds, elongation at 72°C for 10 seconds, after which a final extension of 2 minutes at 72°C. The PCR products were cleaned-up using the Monarch® PCR and DNA clean-up kit (New England Biolabs, USA). The PCR reactions were mixed with 100 µl of the DNA Clean-Up Binding Buffer, transferred to the Clean-Up Columns and centrifuged at 13000 rpm for 1 minute. The bound DNA was washed with DNA Wash Buffer and centrifuged at 13000 rpm for 1 minute, twice, followed by elution in 20 µl molecular grade water (Sigma, UK) by centrifuging at 13000 rpm for 1 minute after incubating at room temperature for 2 minutes. All PCR products were Sanger sequenced at Source Biosciences.

Statistical analysis

Statistical analysis of the growth of *E. coli* 10129 in LB broth, Iso-sensitest broth and M9 and the competitive fitness of the six resistant isolates in LB broth was performed using Ordinary One-Way

ANOVA in GraphPad Prism (version 7) and all other statistical analysis was performed using 2-way ANOVA, also in GraphPad Prism (version 7).

Results

Selection of E. coli 10129 resistant isolates

The MIC of AMC to *E. coli* 10129 was initially determined to be 8 µg/ml. To select for isolates showing decreased susceptibility to AMC, *E. coli* 10129 was grown in the presence of sub-inhibitory concentrations (4 µg/ml) of AMC in defined (M9), semi-defined (Iso-sensitest broth) and undefined (LB broth) media.

There was no significant difference in the cell densities following growth of *E. coli* 10129 in the absence of AMC between three different media types (P value = 0.1071, Fig. 1a) and therefore the cell density of the inoculum was also comparable (P value = >0.9999). Growth was visible between LB and Iso-sensitest media in the presence of AMC however a reduction in cell density was observed following incubation in M9 containing AMC (Fig. 1b) and it was not possible to recover any isolates from agar containing 8 or 16 µg/ml AMC (Table 1). Unlike M9, following exposure of *E. coli* 10129 to AMC in Iso-sensitest broth there was no difference in CFU/ml (P value = 0.3325, Fig. 1b) and we could recover isolates that showed reduced susceptibility to AMC (Table 1). There was significant growth of *E. coli* 10129 of 1.03 log CFU/ml in LB broth in the presence of AMC compared to the initial inoculum (P value = 0.0422) and colonies were recovered at the highest concentration of AMC tested. Therefore, 10 colonies were selected from agar plates containing 8 µg/ml AMC and 10 from plates containing 16 µg/ml AMC following growth in Iso-sensitest and LB broth, respectively.

MIC of AMC resistant E. coli *10129*

Of the 10 resistant isolates of *E. coli* 10129 grown in either LB or Iso-sensitest, three from each media-type were selected at random. The three isolates selected following growth in Iso-sensitest

broth were designated ISO_2, ISO_9 and ISO_10 and those grown in LB broth were designated LB_1, LB_2 and LB_5.

Reduced susceptibility to AMC of the resistant isolates were confirmed through MIC determination and comparison to the ancestral isolate (Table 2) and all were above the clinical definition for resistance according to the EUCAST guidelines for systemic infections caused by *E. coli* (>8 µg/ml). While there was within media-type variability in the increase in AMC MIC of the resistant isolates, there was a clear distinction between those resistant isolates from LB broth and those from Iso-sensitest broth. There was a 1 to 3-fold increase in the AMC MIC in resistant isolates from Iso-sensitest broth, whereas those recovered following growth in LB broth resulted in a 3 to 15-fold increase in MIC. The most resistant isolates were determined to be LB_2 and LB_5, both with a 7 to 15-fold increase in MIC in comparison to the ancestral isolate.

Competitive fitness of AMC resistant isolates

The relative fitness of the resistant *E. coli* 10129 derivatives was initially assessed in LB broth. It was found that the resistant isolates isolated from derived during the Iso-sensitest incubation all incurred a high, but variable, relative fitness cost in comparison to the ancestral isolate, ranging from -4.9 to -20 (Fig. 2). Resistant isolates generated during incubation in LB broth also varied in fitness, with the LB_1 resistant isolate incurring a relative fitness cost of -4 while LB_2 and LB_5 were fitter with a relative fitness of 0.12 and 0.47, respectively (Fig. 2). It is worth mentioning that both isolates incurred the highest increase in MIC compared to the ancestral strain (Table 2).

To determine whether the relative fitness of a particular isolate differs in different media, which has important implications for translation of results to clinical situations, we compared the competitive fitness of the two fitter isolates, LB_2 and LB_5, in LB broth, Iso-sensitest broth and M9. There was no significant difference in relative fitness when the same individual isolate was assessed in both LB

broth and Iso-sensitest (P value = >0.9999) and no significant difference between the relative fitness of LB_2 and LB_5 when compared in LB broth (P value = 0.9992) or Iso-sensitest broth (P value = >0.9999). The relative fitness of LB_2 was significantly different when assessed in M9 media compared to its growth in Iso-sensitest broth (P value = <0.0001) and LB broth (P value = <0.0001). Additionally, there was a significant difference in relative fitness between LB_2 and LB_5 when assessed M9 media (P value = <0.0001).

MIC of E. coli 10129 in different media

To determine if the observed differences in fitness were due to differences in susceptibility in different media we carried out MIC determination for the ancestral strain in the three-different media which we used for generation of resistance (at a concentration of 4-8 µg/ml). We found that, while the MIC was comparable to that determined in MHB for LB and Iso-sensitest grown strains, the MIC in M9 was lower at 1-2 µg/ml.

Investigation of the ampC *promoter region*

Chromosomal mutations of *E. coli* that lead to resistance to β-lactams are often found within the promoter region of the *ampC* gene, resulting in over production of the β-lactamase AmpC [*16-18*]. Two resistant isolates from LB broth and which are fitter than the ancestral strain; LB_2 and LB_5, contained a single mutation at nucleotide position -32 in the -35-box promoter region (Fig. 4). This mutation converts the weak -35 promoter (TTGTCA) to a stronger promoter (TTGACA) [*19*] which has been previously linked to 21-fold increase in AmpC production [*20*].

Discussion

The prospect of using reproducible evolutionary trajectories to resistance as a tool to control the emergence and persistence of AMR is a growing area of research [*2, 3*]. One of the prerequisites for

translation of these ideas to the clinic in the form of prescription policy advice is robust and reproducible data which can convince clinicians and policy makers that what is seen in the laboratory is likely to occur in patients. Therefore, there is a need to analyse the consequences of different experimental conditions thoroughly.

In this preliminary study we have easily identified conserved, previously reported, evolutionary trajectories to AMC resistance (i.e. mutations in the *ampC* promoter) whilst at the same time have shown that there are important differences in the behaviour of strains, both pre, and post selection, when grown in different media.

The most obvious difference is that the MIC of *E. coli* 10129, when grown in M9, is much lower than when grown in all other media and this is likely to have precluded the generation of resistant derivatives. Despite this when we analysed the fitness of AMC resistant isolates, which were generated in either LB or Iso-sensitest media we were also able to show striking differences in the fitness of strains when fitness assays were carried out in different media, most noticeably the unexpectedly high fitness cost of the LB_2 isolate when grown in M9.

Disparities in the fitness of a single resistant isolate in two separate media types has highlighted the potential importance of assessing competitive fitness in different environments. Although both LB_2 and LB_5 contain the same mutation in the *ampC* promoter region which confers resistance to AMC [*19, 20*], it is a likely that either LB_5 has a compensatory mutation which allows it to grow better in M9 or there is a mutation elsewhere within the genome of the strains which results in a negative (for LB-2) or positive epistatic (for LB-5) interaction(s) which only affect growth in M9. It is noteworthy that any compensatory mutations, or mutations resulting in epistatic interactions identified in the genome would not necessarily be ascribed to the phenotype if comparative fitness assays were not carried out in these different media. Although a previous meta-analysis of the

assessment of mean fitness costs of antimicrobial resistance has shown that there is no difference between *in vitro* and *in murine* models [*21*] our observations suggest is would be prudent to consider multiple growth media when extrapolating laboratory acquired data to the clinic and more *in vivo* situations of antibiotic exposure and bacterial growth.

Acknowledgements

This work was supported by the Medical Research Council through the Scheme; Antimicrobial Resistance in a Global Context – A Cross-Council call in partnership with the Department of Health (Grant number; MR/R015074/1).

References

1. Singer AC, Shaw H, Rhodes V*, et al.* Review of Antimicrobial Resistance in the Environment and Its Relevance to Environmental Regulators. Front Microbiol. 2016:7(1728.DOI:10.3389/fmicb.2016.01728.
2. Wong A. Epistasis and the Evolution of Antimicrobial Resistance. Front Microbiol. 2017:8(246.DOI:10.3389/fmicb.2017.00246.
3. Hughes D and Andersson DI. Evolutionary Trajectories to Antibiotic Resistance. Annu Rev Microbiol. 2017:71(579-96.DOI:10.1146/annurev-micro-090816-093813.
4. Suzuki S, Horinouchi T, and Furusawa C. Acceleration and suppression of resistance development by antibiotic combinations. BMC Genomics. 2017:18(1):328.DOI:10.1186/s12864-017-3718-2.
5. Palmer AC, Toprak E, Baym M*, et al.* Delayed commitment to evolutionary fate in antibiotic resistance fitness landscapes. Nat Commun. 2015:6(7385.DOI:10.1038/ncomms8385.
6. Knöppel A, Näsvall J, and Andersson DI. Evolution of Antibiotic Resistance without Antibiotic Exposure. Antimicrobial Agents and Chemotherapy. 2017:61(11): e01495-17 DOI:10.1128/AAC.01495-17.
7. Westhoff S, Van Leeuwe TM, Qachach O*, et al.* The evolution of no-cost resistance at sub-MIC concentrations of streptomycin in *Streptomyces coelicolor*. ISME J. 2017:11(5):1168-78.DOI:10.1038/ismej.2016.194.
8. Gullberg E, Cao S, Berg OG*, et al.* Selection of resistant bacteria at very low antibiotic concentrations. PLoS Pathog. 2011:7(7):e1002158.DOI:10.1371/journal.ppat.1002158.
9. Starikova I, Al-Haroni M, Werner G*, et al.* Fitness costs of various mobile genetic elements in *Enterococcus faecium* and *Enterococcus faecalis*. J Antimicrob Chemother. 2013:68(12):2755-65.DOI:10.1093/jac/dkt270.
10. Olivares Pacheco J, Alvarez-Ortega C, Alcalde Rico M*, et al.* Metabolic Compensation of Fitness Costs Is a General Outcome for Antibiotic Resistant *Pseudomonas aeruginosa* Mutants Overexpressing Efflux Pumps. mBio. 2017:8(4):e00500-17 DOI:10.1128/mBio.00500-17.

11. Musicha P, Feasey NA, Cain AK*, et al.* Genomic landscape of extended-spectrum beta-lactamase resistance in *Escherichia coli* from an urban African setting. J Antimicrob Chemother. 2017:72(6):1602-09.DOI:10.1093/jac/dkx058.
12. Farrell DJ, Robbins M, Rhys-Williams W*, et al.* Investigation of the potential for mutational resistance to XF-73, retapamulin, mupirocin, fusidic acid, daptomycin, and vancomycin in methicillin-resistant *Staphylococcus aureus* isolates during a 55-passage study. Antimicrob Agents Chemother. 2011:55(3):1177-81.DOI:10.1128/AAC.01285-10.
13. Lenski RE, Rose MR, Simpson SC*, et al.* Long-Term Experimental Evolution in *Escherichia coli*: Adaptation and Divergence During 2,000 Generations. The American Naturalist. 1991:138(6):1315-41.DOI:
14. Corvec S, Prodhomme A, Giraudeau C*, et al.* Most *Escherichia coli* strains overproducing chromosomal AmpC beta-lactamase belong to phylogenetic group A. J Antimicrob Chemother. 2007:60(4):872-6.DOI:10.1093/jac/dkm284.
15. Peter-Getzlaff S, Polsfuss S, Poledica M*, et al.* Detection of AmpC beta-lactamase in *Escherichia coli*: comparison of three phenotypic confirmation assays and genetic analysis. J Clin Microbiol. 2011:49(8):2924-32.DOI:10.1128/JCM.00091-11.
16. Siu LK, Lu PL, Chen JY*, et al.* High-Level Expression of AmpC β-Lactamase Due to Insertion of Nucleotides between -10 and -35 Promoter Sequences in *Escherichia coli* Clinical Isolates: Cases Not Responsive to Extended-Spectrum-Cephalosporin Treatment. Antimicrobial Agents and Chemotherapy. 2003:47(7):2138-44.DOI:10.1128/aac.47.7.2138-2144.2003.
17. Tracz DM, Boyd DA, Bryden L*, et al.* Increase in ampC promoter strength due to mutations and deletion of the attenuator in a clinical isolate of cefoxitin-resistant *Escherichia coli* as determined by RT-PCR. J Antimicrob Chemother. 2005:55(5):768-72.DOI:10.1093/jac/dki074.
18. Tracz DM, Boyd DA, Hizon R*, et al.* ampC gene expression in promoter mutants of cefoxitin-resistant *Escherichia coli* clinical isolates. FEMS Microbiol Lett. 2007:270(2):265-71.DOI:10.1111/j.1574-6968.2007.00672.x.
19. Caroff N, Espaze E, Gautreau D*, et al.* Analysis of the Effects of -42 and -32 ampC Promoter Mutations in Clinical Isolates of *Escherichia coli* Hyperproducing AmpC. Journal of Antimicrobail Chemotherapy. 2000:45(783-88.DOI:
20. Jaurin B, Grundstrom T, and Normark S. Sequence elements determining ampC promoter strength in *E. coli.* The EMBO Journal 1982:1(7):875-81.DOI:
21. Vogwill T and Maclean RC. The genetic basis of the fitness costs of antimicrobial resistance: a meta-analysis approach. Evol Appl. 2015:8(3):284-95.DOI:10.1111/eva.12202.

Tables and Figures

Table 1: Recovery of AMC resistant colonies of *E. coli* 10129 on LB agar containing either 8 µg/ml (MIC) or 16 µg/ml (2xMIC) AMC following 24-hour growth in M9, LB broth or Iso-sensitest broth containing sub-inhibitory concentrations of AMC (4 µg/ml). -; 0 CFUs, +; 1-300 CFUs, ++; >300 CFUs.

Table 2: MIC of *E. coli* 10129 and resistant derivatives.

Figure 1: Difference in log CFU/ml of *E. coli* 10129 (a) after growth in LB broth, Iso-sensitest broth and M9 and (b) following 24-hour exposure with sub-inhibitory concentrations of AMC in LB broth, Iso-sensitest broth and M9 compared to the initial inoculum. Error bars represent standard error of the mean.

Figure 2: Relative fitness of selected mutants of *E. coli* 10129 isolated following growth in sub-inhibitory concentrations of AMC in either Iso-sensitest or LB broth compared to ancestral isolate (WT_10129). Error bars represent standard error of the mean

Figure 3: Relative fitness of LB_2 and LB_5 mutants of *E. coli* 10129 isolated following growth in sub-inhibitory concentrations of AMC in LB broth, in LB broth, Iso-sensitest broth and M9, compared to ancestral isolate (WT_10129). Error bars represent standard error of the mean

Figure 4: ampC promoter region, comprising of the -35 and -10 regions of the ancestral isolate (*E. coli* 10129) and six selected AMC resistant mutants isolated following 24-hour exposure in either Iso-sensitest or LB broth.

Table 1

***E. coli* 10129**	**M9 Media**	**LB Broth**	**Iso-sensitest Broth**
LB agar + 8 µg/ml	-	++	++/+
LB agar + 16 µg/ml	-	++	-

Table 2

E. coli 10129	**ISO_2**	**ISO_9**	**ISO_10**	**LB_1**	**LB_2**	**LB_5**
4-8 µg/ml	16 µg/ml	8-16 µg/ml	8-16 µg/ml	32 µg/ml	64 µg/ml	64 µg/ml

Fig. 1a

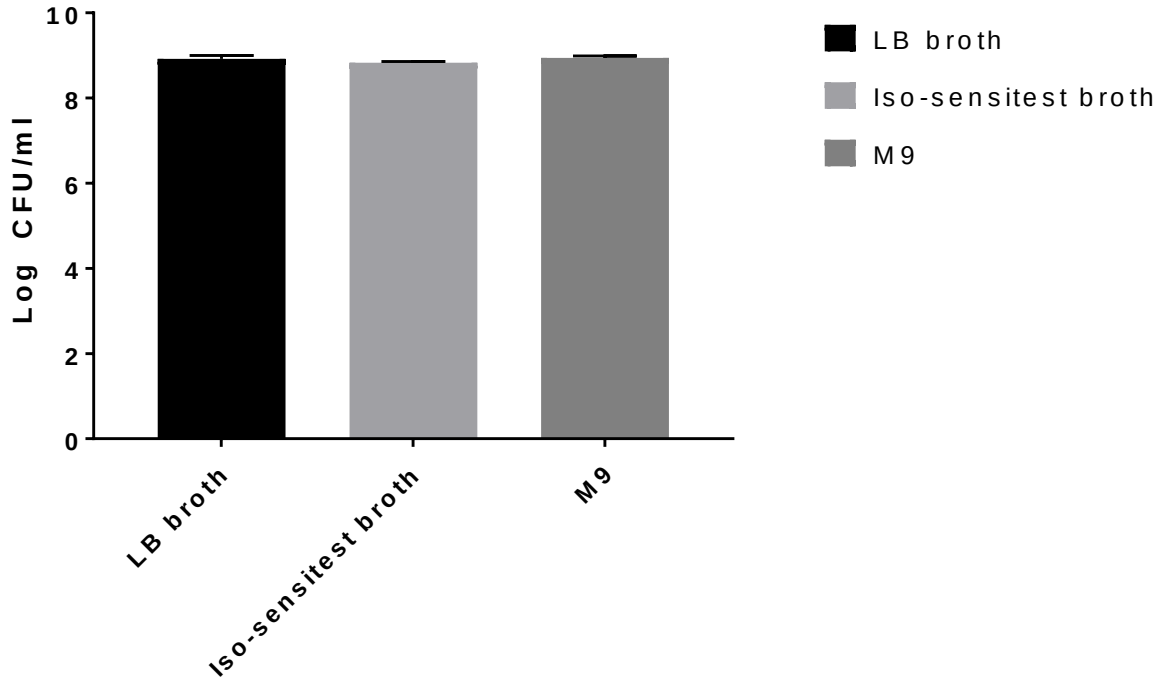

Fig. 1b

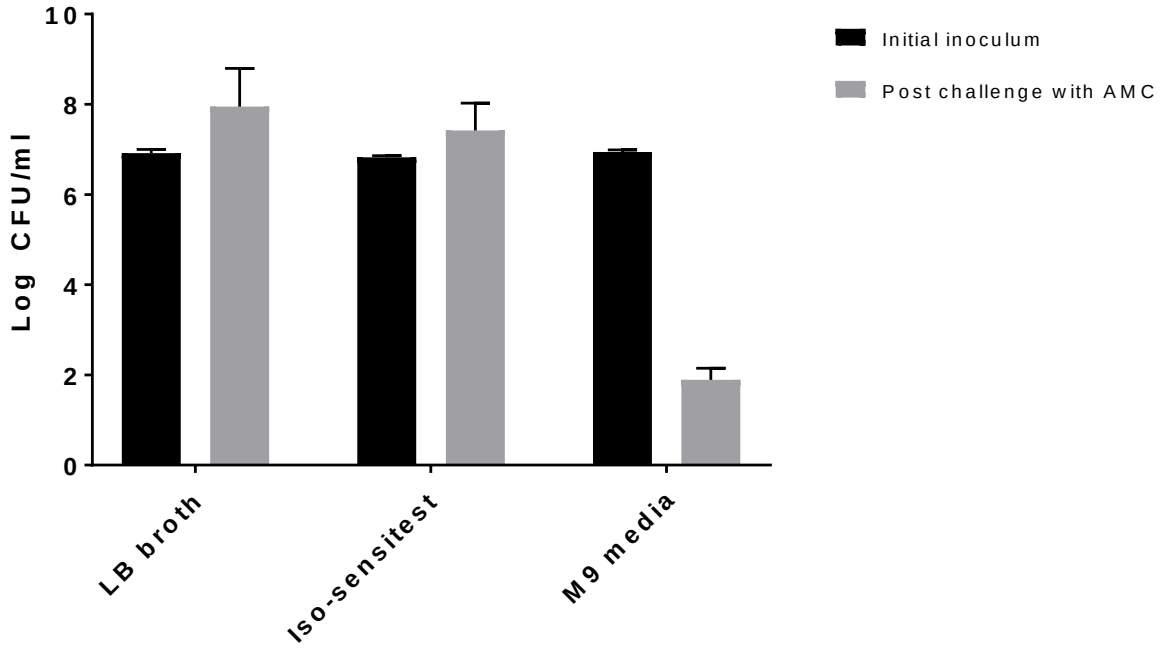

Fig. 2

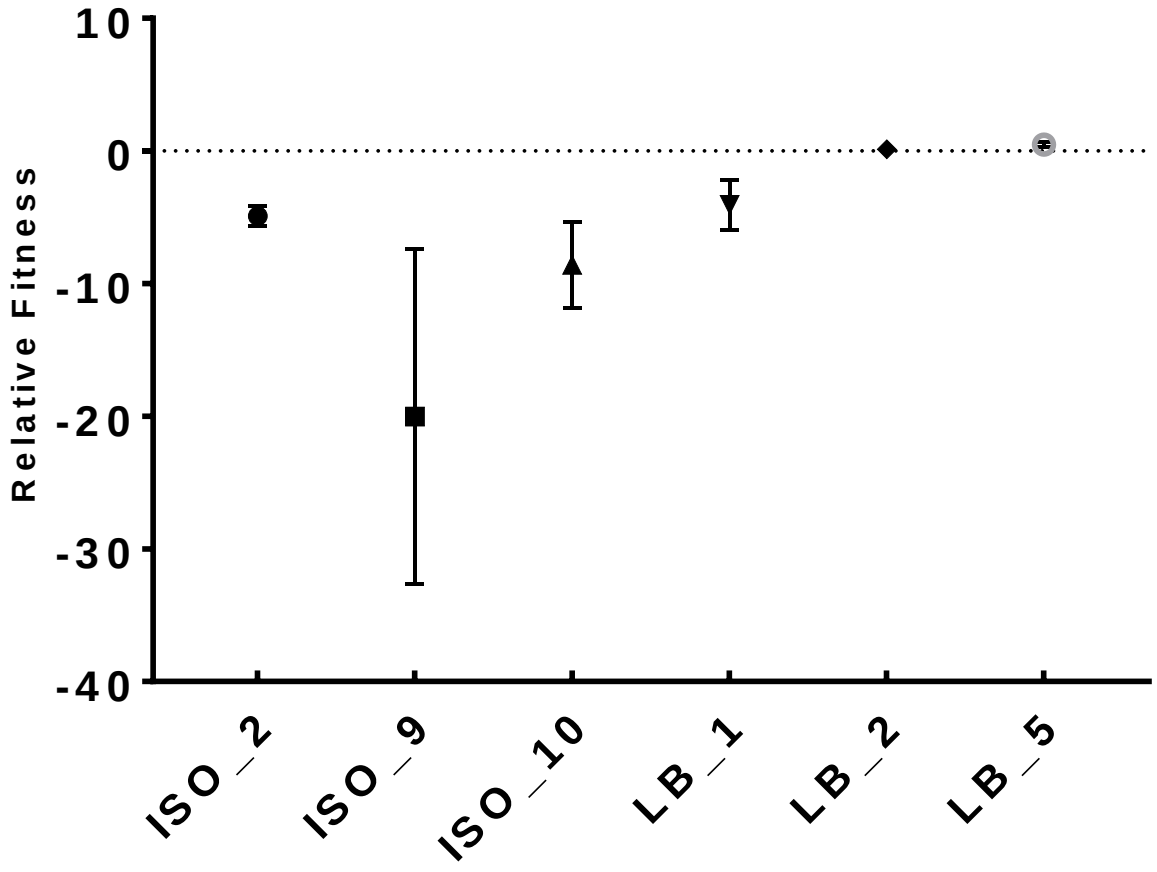

Fig. 3

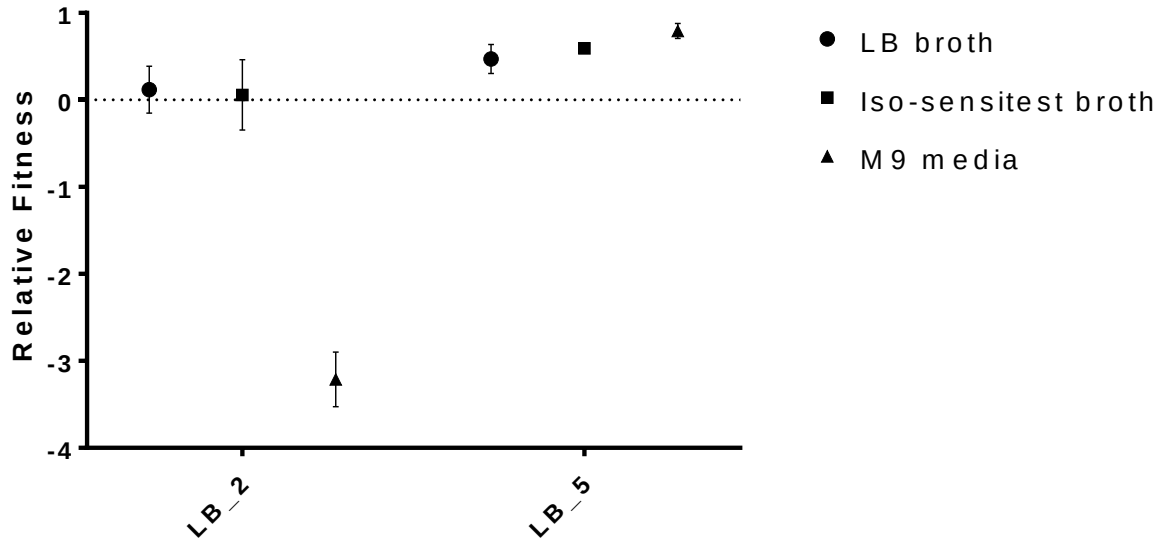

Fig. 4