Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

**The Faculty of Natural Resources and
Agricultural Sciences**

De novo resistance mutations at sub-MIC levels

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Abstract

Antibiotic resistance is a kind of achievable drug resistance meaning that microorganisms are able to sustain and survive exposure to antibiotics. A major contribution to development of antibiotic resistance is the abuse of antibiotics in human and in veterinary medicine. Antibiotics have been used for many decades but only during recent years studies have been published that have increased the understanding in the area of antibiotic input and consequences thereof on bacterial resistance. Thus, it was just recently published that bacterial strains can undergo rapid selection of resistant mutants at antibiotic concentrations far below MIC (Minimal Inhibitory Concentration). However, presently there are no studies at low antibiotic concentrations that have been able to show whether growth and development of *de novo* resistant mutants competitively can out grow the normal bacterial population. Therefore, the aim of this project was to investigate whether *de novo* resistant mutants of *Salmonella typhimurium* and *Escherichia coli* can appear and take over the normal bacterial population, and if this occurs also to isolate the resistant mutations for characterization. These are closely related bacteria but they differ in their target sites for antibiotic resistance i.e. *S. typhimurium* carries a cryptic aminoglycoside resistance gene, *aadA*, that can be up-regulated and thereby causing streptomycin resistance, while the most common low-cost mutations conferring resistance to ciprofloxacin in *E. coli* are in the gyrase gene *gyrA*.

The results showed that concentrations of antibiotics far below MIC can select for *de novo* mutants with high antibiotic resistance both in *S. typhimurium* and *E. coli*. The novel findings regarding these resistant mutants are that they are likely to have lower fitness cost than previously studied resistance mutations (such as rpsL mutants for streptomycin) which means that these mutations may be new and different from those previously described.

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Introduction

Antibiotic resistance is a kind of achievable drug resistance meaning that microorganisms are able to sustain and survive exposure to antibiotics. A major contribution to development of antibiotic resistance is the abuse of antibiotics in human and in veterinary medicine (Martinez, 2008). For instance, the misuse and overuse of prescribed antibiotics by physicians as well as by patients suffering from non-relevant diseases have resulted in a global over consumption of antibiotics. In some countries it is even possible to buy antibiotics without any prescription. Antibiotics are also used in veterinary medicine, including in animal food for preventing diseases and to increase growth. An estimated use of antibiotics in the USA showed that almost 70% of the animal food contains antibiotics, and it was suspected that the use of antibiotics in animal food is closely related to the development of resistant microorganisms and increased incidence of infection-induced diseases (Witte, 1998).

The main reason for development of antibiotic resistance in bacteria, are mutations in specific genes. During optimal conditions a bacterial population approximately doubles in number every 30 minutes and it can reach a population density of more than 10^9 cfu/ml. However, during replication mutations occur frequently, resulting in 10^5-10^6, gene mutations in each millilitre of the bacterial culture. These mutations often occur as silent mutations or are present in non coding regions. However, in some cases the mutation leads to lethal alterations in the bacteria or permits selection of new phenotypes that make the organism fitter for growth and survival, including for example development of antibiotic resistance (Mobashery *et al*., 2002).

Over the past years, understanding of the mechanisms behind development of antibiotic resistance has become an issue of outmost importance for human health and survival. Only a few main mechanisms are known to be able to induce bacterial drug resistance. In order to prevent uptake of drugs into the cellular environment, the bacteria can alter the uptake or efflux system for the drug or could change the composition of their cell membrane. If the drug successfully enters the bacteria it may be modified by cellular metabolizing enzymes, rendering it inactive. Furthermore, mutations in target sites can on the protein/RNA level change the specificity for binding of antibiotics. Which of these mechanisms occur depends on the bacterial species, target site, and the type of antibiotics. The nature of the antibiotic resistance is also dependent on if mediated by a resistance plasmid or by a chromosomal mutation (Mobashery *et al*., 2002).

Antibiotics have been used for many decades but it is only during recent years that studies covering antibiotic input and effects have been published (Kummerer, 2009). Thus, it was only recently discovered that bacterial strains can undergo rapid selection of pre existing resistant mutants at antibiotic concentrations far below the MIC (Minimal Inhibitory Concentration). The definition of MIC is the lowest concentration of an antibiotic that is able to inhibit growth of the bacteria. This MIC level differs between

different bacterial strains. However, presently there are no studies at low antibiotic concentrations that have been able to show whether *de novo* developed resistant mutants competitively can outgrow the normal bacterial population (Gullberg *et al.,* 2011).

Antibiotic resistance is the main driving force of the selection above MIC. However, development of mutants having antibiotic resistance often results in decreased bacterial fitness, including reduced growth rate and virulence. Since all bacterial competitors will die, selection even of mutations associated with a high cost will occur. This is not the case at sub-MIC concentrations of antibiotics. At lower concentrations of antibiotics competing bacteria will not die but instead decrease their growth rate. As a consequence only mutations where the fitness cost is lower than that of competing bacteria will be successful for selection and enrichment (Maisnier-Patin *et al*., 2002).

The aim of the present project was to investigate whether resistant mutations can appear *de novo* and take over the normal bacterial population and, if this occurs, also to isolate the resistant mutants for characterization. In this experiment two different strains of bacteria were used, *Escherichia coli* MG1655 and *Salmonella typhimurium* LT2. They are closely related bacteria but differ in their target sites for antibiotic influence. *S.typhimurium* carries a cryptic amino glycoside resistance gene, *aadA*, that can be up-regulated and thereby causing streptomycin resistance (Koskiniemi *et al*., 2011), while the most common low-cost mutations conferring resistance to ciprofloxacin in *E.coli* are in the gyrase gene *gyrA* (Karlsson *et al., 2003*).

Methods and materials

Bacterial strains, stock solutions and growth conditions

Strains
Strains that were used for this experiment were *Escherichia coli* MG1655 (DA5438) and *Salmonella typhimurium* LT2 (DA6192). The strains were obtained from the strain collection of The Department of Medical Biochemistry and Microbiology (IMBIM).

The MICs for the susceptible strains were for the *S. typhimurium* wild type 4 µg/ml (streptomycin) and for the *E. coli* wild type 23 ng/ml (ciprofloxacin).
S. typhimurium was cycled with streptomycin at ¼ and 1/10 of MIC, and *E. coli* was cycled in ciprofloxacin at 1/10 and 1/50 of MIC. The MIC concentrations for *S. typhimurium* and *E. coli* were provided by my supervisor Dan I. Andersson.

Stock solutions
Streptomycin stock solution (200mg/ml)
A stock solution was prepared from 1000 mg streptomycin mixed with 5 ml water. The new stock solution was aliquoted and filter sterilized into five 1.5 ml tubes. Thereafter the tubes were stored at -20°C.

Streptomycin stock solution (4 mg/ml)
From the stock solution of streptomycin (200 mg/ml) 200 µl was mixed with 9.8 ml water. The new stock solution was aliquoted and filter sterilized into ten 1.5 ml tubes and thereafter stored at -20°C until use.

Ciprofloxacin stock solution (9.2 mg/ml)
A stock solution was prepared from 73.6 mg ciprofloxacin and mixed with 8 ml 0.1M NaOH in water. The stock solution was aliquoted and filter sterilized into 1.5 ml tubes and thereafter stored at -20°C until use.

Ciprofloxacin stock solution 20x (460 µg/ml)
From the stock solution of ciprofloxacin (9.2 mg/ml) 500 µl was mixed with 9.5 ml water. The new stock solution was aliquoted and filter sterilized into 1.5 ml tubes and thereafter stored at -20°C until use.

Ciprofloxacin stock solution 800x (11.5 µg/ml)
From the stock solution of ciprofloxacin (460 µg/ml) 250 µl was mixed with 10 ml water. The new stock solution was aliquoted and filter sterilized into Eppendorf tubes and thereafter stored at -20°C.

Liquid growth medium
The medium used was Mueller-Hinton broth (Becton Dickinson, MD, USA). It was prepared by mixing 4.2 gram Mueller-Hinton media with 200 ml water and thereafter autoclaved.

In the experiments used for growing the bacterial strains Mueller-Hinton medium was used that contained different concentrations of streptomycin (1.0 μg/ml and 0.4 μg/ml) and ciprofloxacin (2.3 ng/ml and 0.46 ng/ml). The strains were grown at 37°C on a shaking board.

LB-agar plates
LB-agar plates were used as a solid medium for screening for resistant the mutants every tenth day. For the preparation of plates 20 gram of LB-agar powder (Sigma-Aldrich, MO, USA) was mixed with 500 ml water, autoclaved and poured into petri-dishes.
LB-plates were made that contained different concentrations of streptomycin (8 μg/ml, 16 μg/ml, 32 μg/ml, 64 μg/ml, 96 μg/ml and 128 μg/ml) and ciprofloxacin (46 ng/ml, 92 ng/ml, 138 ng/ml and 184 ng/ml).

Growth media containing streptomycin 0.4 μg/ml and 1.0 μg/ml
From the streptomycin stock solution (4 mg/ml) 10 μl was transferred into an 1.5 ml tube containing 0.99 ml Mueller-Hinton medium. The solution was vortexed and 240 μl and 600 μl was transferred into 50 ml tubes containing 23.76 and 23.40 ml, respectively, to reach the final streptomycin concentrations of 0.4 and 1.0 μg/ ml.

Growth media containing ciprofloxacin 0.46 ng/ml and 2.3 ng/ml
From the ciprofloxacin stock solution (11.5 mg/ml) 10 μl was transferred into an 1.5 ml tube containing 0.99 ml Mueller-Hinton medium. The solution was vortexed and 96 μl and 480 μl was transferred into 50 ml tubes containing 23.90 ml and 23.52 ml, respectively, to reach the final ciprofloxacin concentrations of 0.46 and 2.3 ng/ml.

Cycling the bacteria in growth medium
The bacterial strains from the -80°C storage were streaked on pre-warmed (37°C) LB-plates and incubated over night at 37°C. The following day one colony was picked with a sterile loop and inoculated into a 10 ml tube containing 1 ml Mueller-Hinton medium. The tube was incubated over night on a shaking board at 190 rpm and 37°C. 24 hours later the pre culture was diluted by firstly transferring 10 μl from the pre culture to a new 10 ml tube containing 1 ml Mueller-Hinton. From this tube 1 μl was transferred to a new 10 ml tube containing 1 ml Mueller-Hinton medium (total dilution 1:10,000) containing streptomycin or ciprofloxacin (see the above medium section).

Each day of the experiments 1 μl from the overnight culture was transferred into a new 10 ml tube containing 1 ml Mueller-Hinton medium with the different concentrations of streptomycin or ciprofloxacin. The tubes were incubated on a shaking board at 37°C over night.

Every third day during the first 15 days of the experiments, 900 μl of the overnight cultures were frozen in -80°C in a freezing tube containing 100 μl dimethyl sulfoxide (DMSO). Thereafter, samples were frozen once a week. In order to screen for resistant mutants, 1000x dilutions of the overnight cultures were every tenth day spread on pre-

warmed LB-plates containing different concentrations of streptomycin or ciprofloxacin, (see LB-agar plates). To be able to compare the results of development of antibiotic resistance also cultures of the wild-type of both bacterial strains were spread out on LB-plates containing streptomycin or ciprofloxacin.

When mutants with high antibiotic resistance started to appear, colonies were picked and restreaked on new plates containing the same antibiotic concentrations to check whether persistent resistance had been obtained. Thereafter, these colonies were transferred to 10 ml tubes containing 1 ml of Mueller-Hinton-medium with the same concentrations of antibiotics that were used when cultures were cycled. The tube was placed in the incubator over night and thereafter transferred into a freezing tube containing DMSO and frozen in -80°C.

Results

During the experiments twenty lineages of each strain (*E. coli* and *S. typhimurium*), for each concentration were used making a total of 100 lineages. Every third day, cultures of the lineages were frozen at -80°C, and for every tenth cycle a screening for resistant mutants was made on agar plates containing the antibiotics (streptomycin and ciprofloxacin). The results from these experiments are shown in Figures 1 and 2.
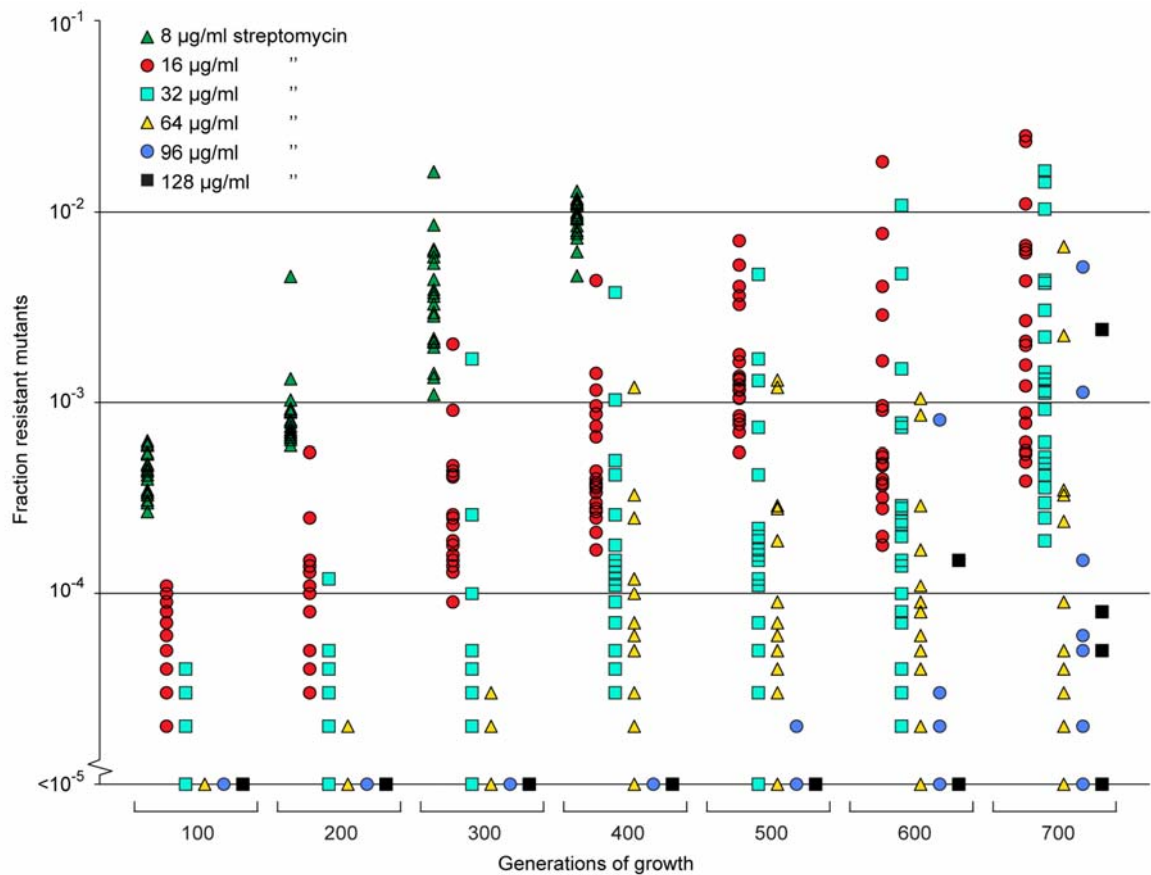
Figure 1. Selection of *de novo* resistant mutants of *S.typhimurium* at sub-inhibitory concentrations of streptomycin. Twenty independent lineages of *S.typhimurium* were serially passaged in Mueller-Hinton medium containing 1 µg/ml streptomycin. At every 100 generations a screening for mutants was made on LB-plates containing different concentrations of streptomycin and the proportions of resistant mutants were calculated.
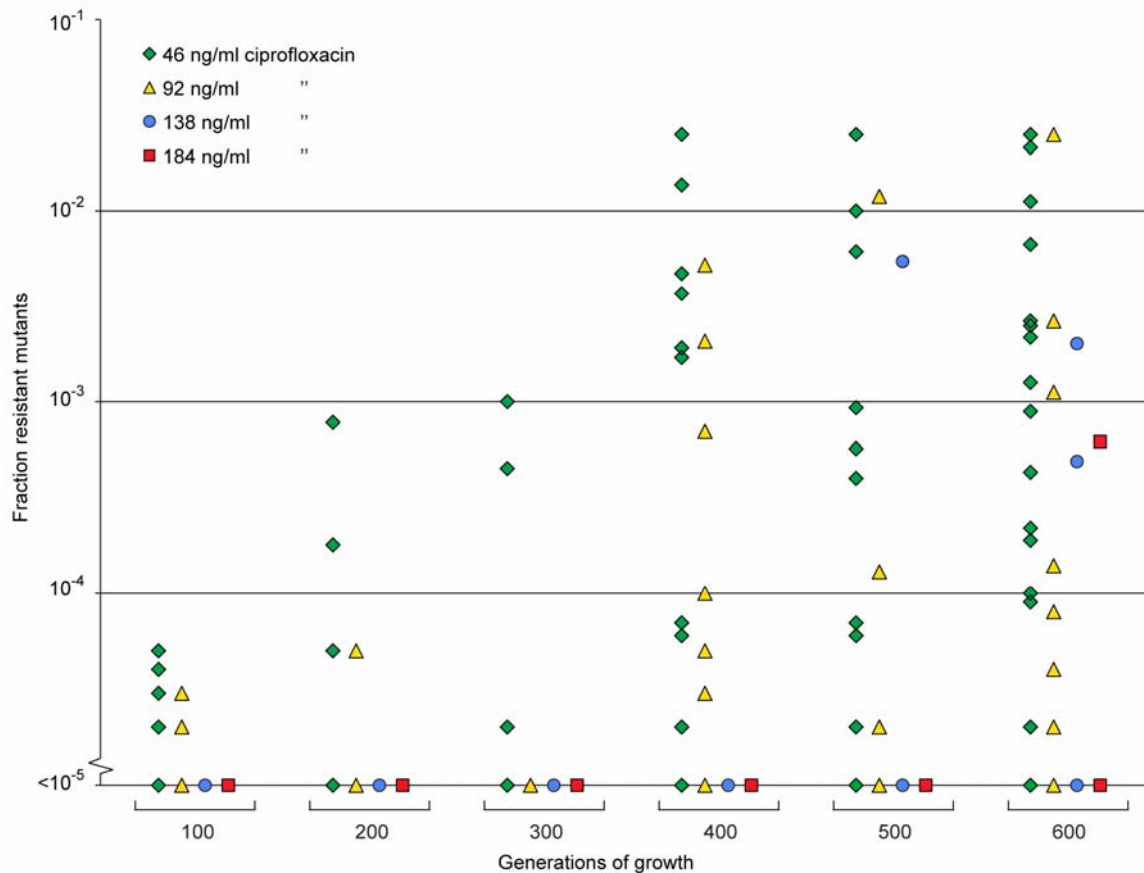
Figure 2. Selection of *de novo* resistant mutants of *E. coli* at sub-inhibitory concentrations of ciprofloxacin. Twenty independent lineages of *E. coli* were serially passaged in Mueller-Hinton medium containing 2.3 ng/ml ciprofloxacin. At every 100 generations a screening for mutants were made on LB-plates containing different concentrations of ciprofloxacin and the fractions of resistant mutants were calculated.

Comparable experiments as reported in Figures 1 and 2 were performed in *S. typhimurium* that was grown in 0.4 μg/ml streptomycin and *E. coli* that was grown in 0.46 ng/ml ciprofloxacin. In these experiments, however, no enrichment of resistant mutants could be observed.

To be able to compare the results in Figures 1 and 2 with the controls without added antibiotics of *S. typhimurium* and *E. coli*, overnight culture were accordingly spread out on plates containing streptomycin and ciprofloxacin.
When comparing the background of the controls without added antibiotics with the fraction of resistant mutants a clear increase in number of mutants was shown to develop for both *S. typhimurium* (1.0 μg/ml streptomycin) and *E. coli* (2.3 ng/ml ciprofloxacin).

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Discussion

The aim of this project was to investigate whether antibiotic resistant mutations with high fitness and resistance, could appear *de novo* and subsequently take over in bacterial populations of *S. typhimurium* and *E. coli*, and if that occurs to isolate the resistant mutants for further characterization. *S. typhimurium* carries a cryptic aminoglycoside resistance gene, *aadA*, that can be up-regulated and thereby causing streptomycin resistance, while
the most common low-cost mutations conferring resistance to ciprofloxacin in *E. coli* are in the gyrase gene *gyrA* (Koskiniemi *et al.*, 2011, Karlsson *et al., 2003*).

S. typhimurium and *E. coli* that were grown at the higher concentrations of antibiotics (streptomycin 1.0 µg/ml and ciprofloxacin 2.3 ng/ml) enriched resistant mutants more rapidly than the bacteria that were grown at lower concentrations of the antibiotics (streptomycin 0.4 µg/ml and ciprofloxacin 0.46 ng/ml). When comparing *S. typhimurium* and *E. coli* at higher concentrations of the antibiotics, in the *S. typhimurium* cultures mutants started to enrich gradually from the beginning whereas *E. coli* did not show any enrichment of mutants until after 400 generations. An explanation for this could be that streptomycin compared to ciprofloxacin has more potential target sites in the genome for development of antibiotic resistance. Another explanation could be that mutants appeared but none of them were transferred to the new tube during the cycling procedure since only 0.1 % of the cultures were transmitted.

In order to compare the above results that were obtained during the selection of resistant mutants (figure 1 and 2 in results) another experiment was performed using the wt of *S. typhimurium* and *E. coli*. The wt of *S.typhimurium* and *E. coli* were similarly spread out on LB-plates containing different concentrations of the previously tested antibiotics, streptomycin and ciprofloxacin. When the wt background was compared with the fraction of resistant mutants a clear increase was shown for both *S.typhimurium* (1.0 µg/ml streptomycin) and *E.coli* (2.3 ng/ml ciprofloxacin). However, at lower exposures of antibiotics *S. typhimurium* (0.4 µg/ml streptomycin) and *E. coli* (0.46 ng/ml ciprofloxacin) did not show any enrichment of mutants even after 500 generations. A possible explanation for this could be that the used concentrations of the antibiotics and the time of exposure were not sufficient to show enrichment for mutants. This could potentially be answered by using a chemostat experimental setup instead of the performed cycling design. Because new medium are continuously added to the culture no bottleneck occurs. Mutants that appear will be selected and by time increase in number causing the mutated bacterial population to take over.

These results showed that concentrations of antibiotics far below MIC can select for *de novo* mutants with high antibiotic resistance both in *S. typhimurium* and *E. coli*. The novel findings regarding these resistant mutants are that they are likely to have lower fitness cost than previously known resistance mutations (such as rpsL mutants) which means that these mutations may be new and different from those previously described (Koskiniemi *et al.*, 2011).

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In conclusion, these experiments show that concentrations of antibiotics far below MIC can select for *de novo* mutants harboring a high antibiotic resistance. Therefore, further studies should be performed that will focus on these isolated resistant mutants with the aim to identify and characterize the resistance mutations by using transposon mapping and whole-genome sequencing.

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