The effect of environmental pollutants and food processing on the development of antibiotic resistance

Lucia Birosova^a, Maria Mikulasova^b

Aim. The purpose of this study was to determine how various compounds known to be positive mutagens, contribute to the development of mutations leading to ciprofloxacin resistance in *Salmonella enterica* subsp. *enterica* serotype Typhimurium. The molecular mechanism of ciprofloxacin resistance in treated strains was investigated.

Methods. A modified version of the incorporation plate test was used for quantitative determination of ciprofloxacin resistant mutants and for assessment of the mutation frequency induced by the positive mutagens in different concentrations. An AS-PCR-RFLP for monitoring of *gyrA* mutations was applied.

Results. Mutation frequency, expressed as number of antibiotic resistant colonies per 10⁸ viable cells, was much higher after exposure of bacterial cells to 3-(5-nitro-2-furyl) acrylic acid and 2-nitrofluorene. All isolated cultures retain decreased susceptibility to antibiotic after multiple passages in antibiotic-free medium. 2-nitrofluorene was the best inducer of mutations in *gyrA* and in regulation genes affecting suppression of synthesis of outer membrane porins. 3-(5-nitro-2-furyl) acrylic acid gives rise to overproduction of efflux pump.

Conclusions. The data suggest that antibiotic resistance may not be only a consequence of misuse of antibiotics. A polluted environment as well as food processing could contribute to this unwanted process.

Key words: ciprofloxacin, mutagens, resistance, *Salmonella*

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^aDepartment of Nutrition and Food Assessment, Faculty of Chemical and Food Technology, Slovak University of Technology, Bratislava, Slovak Republic

^bDepartment of Molecular Biology, Faculty of Natural Sciences, Comenius University, Bratislava, Slovak Republic
Corresponding author: Lucia Birosova, e-mail: lucia.birosova@stuba.sk

INTRODUCTION

The necessity to retain the efficiency of antibiotics and curtail the expansion of antibiotic resistance has already overwhelmed the scope of health care systems and has become a serious socioeconomic problem.

Bacterial resistance could be caused by horizontal gene transfer, recombination processes and also by genome mutation. Although antibiotic resistance has been studied intensively, mechanisms that generate resistance mutations are poorly understood. Chromosomal mutations can lead to antibiotic resistance in several different ways. In recent years, much attention has been given to the idea that the mutation process in bacterial populations is not a static event but a complex network of factors influencing the rate and the type of mutants¹. Different stresses such as starvation, oxidation or UV irradiation can increase the mutation rates of bacteria². All factors affecting the mutability of chromosomal genes confer resistance among different bacterial species³. As suggested by Martinez and Baquero¹ mutation frequencies are probably much higher in the course of infection than those determined *in vitro*, because bacteria growing *in vivo* are frequently under environmental stress and challenges. Pathogenic bacteria are exposed to different chemical and physical agents which may damage DNA resulting in mutations.

Salmonella is one of the primary causes of bacterial foodborne infections in humans causing illnesses and fatalities all over the world each year. Some of these infections result in bacteraemia requiring treatment with antibiotics⁴. Fluoroquinolones have been used successfully to treat salmonellosis and are the commonly recommended agent of first choice but there is evidence of an increasing incidence of strains that exhibit resistance or decreased susceptibility to the most recent fluoroquinolones⁵⁻⁸. Two mechanisms are involved in the development of resistance to fluoroquinolones: alterations in drug targets DNA gyrase or topoisomerase IV (ref.^{9,10}) and decreased cellular accumulation of quinolones¹¹. Mutations causing quinolone resistance occur primarily in a highly conserved region (QRDR) of DNA gyrase and topoisomerase IV genes¹². Mutations affecting the accumulation of quinolones include those that affect the regulatory gene *marA* (multiple antibiotic resistance) (ref.^{13,14}) generally cause decreased expression of the OmpF porin¹⁵ and overexpression of the AcrAB efflux pump¹⁶. These porin and pump changes lead to resistance not only to quinolones but also to a number of structurally unrelated compounds¹⁷.

The importance of *de novo* mutations in the development of antibiotic resistance prompted us to ask how compounds known as positive mutagens contribute to the development of this unwanted process. The aim of this paper was determine the frequency of induced mutations

that may lead to antibiotic resistance as well as to determine the mechanism of ciprofloxacin resistance induced by tested positive mutagens.

MATERIALS AND METHODS

Bacterial strains and drugs

The bacterial strain used in this study *Salmonella enterica*, subs. *enterica* serotype Typhimurium CCM 4763 was received from the Collection of Microorganisms, Masaryk University, Brno (Czech Republic). Mutant strains were prepared. *Staphylococcus aureus* CCM 885 was obtained from the same collection.

Ciprofloxacin was purchased from Merck (Germany). 2-nitrofluorene (2NF, Sigma Aldrich, Germany) and 2-(5-nitro-2-furyl) acrylic acid (5NFAA, Slovafarma, Slovak Republic) were dissolved in dimethylsulfoxid (DMSO, Merck, Germany).

Antimicrobial susceptibility determination

The minimal inhibition concentration (MIC) of antibiotic was determined by the agar dilution method using agar plates containing serial twofold dilutions of drug according to the Clinical and Laboratory Standards Institute guidelines (formerly the National Committee for Clinical Laboratory Standards) (ref.¹⁸), and by micro-suspension assay in 96-well microplates¹⁹. The MIC was defined as the lowest concentration of drug that inhibits visible growth after 24 h of incubation at 37 °C. The MICs reported here represent the arithmetic mean of the means of three separate experiments.

Determination of mutation frequencies to antibiotic resistance

S. enterica ser. Typhimurium strain was grown overnight in antibiotic-free broth with viable cell number around 10^9 mL⁻¹. This culture was divided into 0.1 mL aliquots, then 0.1 mL of positive mutagen and 0.5 mL of phosphate buffer of pH 7.4 was added and cultures were treated with mutagen for 30 min. Later the fresh Luria-Bertani (LB) medium was added and cultures were incubated for 3 h at 37 °C to allow a few cell divisions and protein expression to occur. The number of ciprofloxacin-resistant mutants that emerged in each culture was determined by plating the entire culture on LB agar plates containing a selective concentration of antibiotic. The total number of viable cells was determined by plating an appropriate dilution of cultures on nonselective medium. Colonies on both selective and nonselective plates

were counted after incubation for 2 days. The frequency of resistant mutants (resistance index RI) was expressed as mean number of resistant cells divided by the total number of viable cells per culture. RI factor represents proportion of induced mutation frequency to frequency of spontaneous mutations leading to ciprofloxacin resistance. Data shown here represent the mean of three independent experiments; each experiment was designed in five parallels and statistically evaluated by the Student's t-test.

Subculturing of mutant isolates

From each of 50 cultures growing in selective antibiotic concentration one random mutant colony was picked and passaged in media without antibiotics for ten consecutive days. Following serial culture, the ciprofloxacin MIC of cells collected after the first and last subculture to antibiotics was determined. Strains, which maintained increased values of MIC, were considered to be resistant mutants. The cells from the same colonies on selective plates were also transferred onto medium containing twice of selective concentration of the antibiotic. After 24 h of incubation, surviving cells were transferred onto medium containing twice the prior concentration of ciprofloxacin and this process was repeated serially until no growth occurred.

Detection of *gyrA* mutations by AS-PCR-RFLP assay

AS-PCR-RFLP (allele specific - PCR - restriction fragment length polymorphism) assay was provided according Giraud et al.²⁰. The sequences of all primers used in PCR amplification are shown in Table 1. The forward primer (STGYRA1) and reverse primer (STGYRA-*HinfI*/87) were predicted to produce a 195 bp fragment with a *HinfI* restriction site for determination of mutations in codon 83 and 87 and allele-specific forward primer (AS-81) for determination of mutation on the 81st codon. PCR was performed with hot-start technique in total volume of 50 µL, which contained 25 pmol of primers STGYRA1 and AS-81, 50 pmol of primer STGYRA-*HinfI*/87, 200 µM deoxynucleotide triphosphates, 2 mM MgCl₂ and 0.5 U of *Taq* polymerase (Promega, Madison). After a denaturation step of 15 min at 95 °C (denaturation of bacterial colonies) denaturation of 3 min at 94 °C was performed, followed by amplification performed over 30 cycles, each one consisting of 1 min at 94 °C, 1 min hybridization temperature at 55 °C and 1 min at 72 °C, with a final extension step of 10 min at 72 °C. Primers and free nucleotides were removed with a Qiaquick spin PCR purification kit (Quiagen S.A., France). Restriction enzyme

Table 1. Primers used for PCR amplification and sequencing.

Primer	Sequence
STGYRA1	5'-TGTCCGAGATGGCCTGAAGC-3'
STGYRA12	5'-CGTTGATGACTTCCGTCAG-3'
STGYRA- <i>HinfI</i> /87	5'-ATGTAACGCAGCGAGAATGGCTGCGCCATACGAACGATGGAG-3'
AS-81	5'-GGTAAATACCATCCCCACG-3'

digestion was performed in 10 μL mixture containing 5 μL of PCR product and 5 U of *Hinf*I. Both digested and undigested PCR products were resolved in 3% (w/v) agarose gel for small fragments. DNA ladder (25 - 500 bp) was used as a molecular marker. A PCR product prepared with 2 primers (STGYRA1 and STGYRA2) was used for sequence determination and PCR reaction was performed as described for AS-PCR-RFLP.

Preparation and analysis of outer-membrane protein (OMP)

Expression of OmpF was determined according Giraud et al.²¹ with slight modification. Briefly, bacteria were mechanically disrupted with glass beads, unbroken cells were removed by centrifugation and supernatant was incubated with 1% (v/v) Triton X-100 and 2% (v/v) lauryl sarcosine. After ultracentrifugation SDS-PAGE was performed and proteins were detected after staining with Coomassie Brilliant Blue.

Measurement of ciprofloxacin accumulation

The uptake of ciprofloxacin by bacterial cells was measured according to Hirai et al.²². Ciprofloxacin at a final concentration of 10 $\mu\text{g mL}^{-1}$ was added to the bacterial cell suspension ($A_{570} = 0.7$), and the cultures were incubated at 37 °C with shaking. In the indicated times (0, 5, 10, 15, 25, 35 min), 10 mL of the culture was chilled and the cells were sedimented by centrifugation. The cells were then suspended in 1 mL of saline. The suspension was immersed in boiling water for 10 min and then centrifuged. All drugs could be extracted from the cells by this treatment and it was also confirmed that ciprofloxacin was not inactivated by boiling. The concentration of ciprofloxacin in the supernatants was measured by bioassay using *Staphylococcus aureus*²³. The inhibition zones produced by 20 μL aliquots of each extract were compared with those produced by 20 μL aliquots of different known ciprofloxacin concentrations using nonlinear regression. The accepted standard deviation for all ciprofloxacin uptake results was always $\leq 5\%$ with respect to each mean value of three measurements. Likewise, all the above were assayed in the presence of 30 μM 3,3',4',5-tetrachlorosalicylanilide (TCS), an uncoupler, added in the 10th minute. For confirmation, ciprofloxacin concentrations were also measured by HPLC.

RESULTS

Mutation frequency and susceptibility of isolated mutants

The mutagenic effect of 2NF and 5NFAA in low concentrations is shown in Fig.1. The frequency of mutations leading to ciprofloxacin resistance was increased significantly with increasing concentration of mutagen. In comparison, 5NFAA was a better inducer of ciprofloxacin resistance because the mutation frequency increased up to 400-fold. For 2NF it was only 8-fold over spontaneous background levels with increasing concentrations of mutagen.

After 10 passages in antibiotic-free medium, all isolated strains were able to grow on selective plates with ciprofloxacin. The sensitivity of these strains to ciprofloxacin decreased very moderately; the majority of strains had ciprofloxacin MIC of 0.78 $\mu\text{g mL}^{-1}$ and only 20% of 5NFAA induced mutants were resistant. Some strains had adapted to higher ciprofloxacin concentrations after the serial passaging on medium with increasing antibiotic concentration. The sensitivity to ciprofloxacin had decreased; even in 35% of strains (for both mutagenic compounds) we observed a very high, clinically significant value MIC of ciprofloxacin (25 - 200 $\mu\text{g mL}^{-1}$). According to National Antimicrobial Susceptibility Program²⁴, ciprofloxacin susceptibility limit for *Salmonella* Typhimurium is 4 $\mu\text{g mL}^{-1}$, and bacteria which grow in the presence of ciprofloxacin in these and higher concentration are considered to be resistant.

Mutations in *gyrA* gene

For determination of mutations in the *gyrA* gene associated with quinolone resistance, all strains with ability to grow on selective concentration of ciprofloxacin were examined for mutations at Gly-81, Ser-83 and Asp-87 in the *gyrA* gene by the AS-PCR-RFLP assay according to Giraud et al.¹⁸. Fig. 2 presents representative agarose gel electrophoresis profiles obtained in the assay with the parent strain and resistant isolates.

All 2NF - mutants had a mutation in the QRDR of *gyrA* gene. All resistant strains ($\text{MIC} > 4 \mu\text{g mL}^{-1}$) had mutation in codon Ser-83 while strains with decreased susceptibility had mutation in codon Asp-87 or Ser-83. In 90% of strains generated after 5NFAA effect we determined mutation only in Ser-83 and no other type of mutation was observed.

DNA sequencing of the 500 bp PCR products covering the entire QRDR of *gyrA* of all mutants with mutations in either of these codons revealed base substitutions in the codon Ser-83 from TCC (Ser) to TTC (Phe) and in the codon Asp-87 from GAC (Asp) to AAC (Asn).

Fig. 1. Effect of 2-(5-nitro-2-furyl) acrylic acid and 2-nitrofluorene on frequency of mutations leading to ciprofloxacin resistance.

Fig. 2. AS-PCR-RFLP patterns of representative strains before *HinfI* digestion (1) and after digestion without mutation (2), with mutation in 83 codon (3) and in 87 codon (4), M - DNA marker (25-500 bp).

We found no Gly-81 mutation nor the combination of the mentioned mutations. It is remarkable that mostly mutation in the 83rd codon led to clinically significant values of ciprofloxacin MIC.

Changes in ciprofloxacin uptake

The same strains were analyzed considering OmpF porin production and the efflux pump expression to determine changed antibiotic concentration in cells. We discovered that OmpF proteins were significantly reduced only in the case of 2NF mutant strains. Decreased levels or total absence of OmpF was determined in 88% of isolated strains (Fig. 3, lane 4,5). In strains affected by 5NFAA we

Fig. 3. OMP profiles prepared from parent strain (lane 1), 5NFAA mutant strains with decreased expression OmpF levels (lanes 2, 3) and 2NF mutant strains with decreased levels or absence of OmpF (lane 4, 5). M - Sigma Marker™ Wide molecular weight range (6,2 - 205 kDa).

detected reduced levels of OmpF only in 33% of strains, all rest mutants had levels of OmpF proteins equivalent to parent, ciprofloxacin susceptible strain.

Fig. 4 shows the uptake of ciprofloxacin by parent strain and mutant NF12 (2NF induced mutant) in which increased efflux was detected. Mutant strain accumulated approximately 50% less ciprofloxacin than parent strain. After addition of an energy inhibitor TCS to the cultures, accumulation of ciprofloxacin increased rapidly in both strains. Uptake increase after TCS addition indicated blockage of an energy-dependent ciprofloxacin efflux.

Such course with minor differences was found in 66% of strains generated after exposure to 5NFAA, even in half of them we registered simultaneously decreased levels of OmpF and these entire mutants were resistant to ciprofloxacin. Only 20% of 2NF mutants had efflux pump overproduction. Simultaneously absence of porins was detected. All these strains had only decreased susceptibility to ciprofloxacin.

DISCUSSION

Salmonella Typhimurium is a foodborne pathogen which colonizes the intestine where it may make contact with mutagens present in some food-stuffs or generated during food processing. Accordingly we focused on the effect of two positive mutagens on the development of ciprofloxacin resistance in *Salmonella enterica* subs. *enterica* serotype Typhimurium. 2-Nitrofluorene is a typical carcinogenic nitropolycyclic aromatic hydrocarbon^{25,26} which was detected in diesel exhaust particles²⁷ and also as a major pollutant in the atmosphere²⁸. In our previous study we discovered that this compound induces mutations leading to antibiotic resistance and selects for high level resistant strains. 2-(5-nitro-2-furyl) acrylic acid used to be a wine stabilizer²⁹.

Fig. 4. Uptake of ciprofloxacin by parent strain and resistant mutant NF12 generated after 2NF exposition with and without addition of TCS.

Positive mutagens used in this study differ in the induction of mutations leading to ciprofloxacin resistance. We also found that doses of mutagens effective in the Ames test did not have to correlate with that effective for antibiotic resistance development. 5NFAA in lower concentrations increased the frequency of mutations leading to ciprofloxacin resistance at a higher rate than 2NF.

Mutations at Asp-87 or Ser-83 in the quinolone resistance-determining region of the *gyrA* gene were found in all mutants isolated after 2NF induction. Although mutations at Asp-87 have usually been reported to be concomitant with mutations at Ser-83 (ref.³⁰), we found a single mutation at Asp-87 in 60% of 2NF mutants in gyrase gene. Nor after adaptation did we obtain mutant strains with double mutation in Ser-83 and Asp-87 in *gyrA*. In 5NFAA mutants we detected only one type of point mutation at Ser-83 of the *gyrA*.

Mutations in regulatory genes of MarRAB operon, which regulate expression of efflux pumps and outer membrane porins can also contribute to high-level fluoroquinolone resistance in *Salmonella* Typhimurium³¹. We discovered that mutation at the 83rd codon of the *gyrA* together with absence of *OmpF* was always connected with the development of ciprofloxacin resistance. 5NFAA was the best inducer of efflux pump overproduction; 2NF was very effective in repression of *OmpF* synthesis.

The link between mutagenicity and a clinical parameter such as antibiotic resistance would provide valuable data to help us understand the problem of the development of drug resistance as well as that associated with the interpretation of mutagenic studies. Knowledge of the effects of some environmental factors on the mutation rate can enable us to predict how fast the bacteria will develop antibiotic resistance in a given environment.

CONCLUSIONS

Many studies indicate that the main cause of antibiotic resistance of pathogenic bacteria is overuse or misuse of antibiotics in human and veterinary medicine. Our results show that environmental pollutants as well as compounds generated during food processing may contribute to the development of mutations leading to this unwanted process. Both tested compounds significantly increased mutation frequency leading to ciprofloxacin resistance in *Salmonella* Typhimurium. 2-nitrofluorene was the best inducer of mutations in *gyrA* and in regulation genes affecting synthesis inhibition of outer membrane porins. 3-(5-nitro-2-furyl) acrylic acid gave rise to overproduction of efflux pump.

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