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DEVELOPMENT OF CIPROFLOXACIN RESISTANCE DUE TO CHROMOSOMAL MUTATIONS INDUCED BY 2-NITROFLUORENE

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In this paper we investigated the ability of 2-nitrofluorene to induce mutations leading to antibiotic resistance in quinolone-sensitive strain *Salmonella typhimurium*. After preincubation of bacteria with 2-nitrofluorene, the frequency of mutation to ciprofloxacin resistance was 57 fold higher than in the case of spontaneous mutability. Some of resultant resistant colonies showed a great increase of ciprofloxacin MIC.

INTRODUCTION

Many mutagenic agents appearing in the environment generated in food processing or contained in some drugs, can react with DNA and be responsible for the induction of mutations. 2-Nitrofluorene (2NF) is a nitropolycyclic aromatic hydrocarbon, which is potentially mutagenic and carcinogenic to humans through inhalation, ingestion, and skin contact[1–3] and is mutagenic for bacteria[4].

Beside undoubted role of the mutations in the carcinogenesis, which is intensively studied, mutations are also the primary cause of drug resistance. Chromosomal mutations can lead to antibiotic resistance in several different ways. A single step mutation that altered a ribosomal protein, for example, conferred resistance to streptomycin by presumably altering the antibiotic-binding site[5]. Mutations in regulators or regulatory regions can contribute to the resistance by leading to the overproduction of efflux pumps or the target itself[6].

Emerging resistance to fluoroquinolones in *Salmonella* spp. has been reported both in human and animal cases and is thus threatening to become a serious public health problem[7,8]. Human nontyphoidal *Salmonella* infections are increasingly frequent in developed countries[9], and the emergence of fluoroquinolone-resistant *Salmonella* strains is a serious matter of concern since this class of antibacterial agents constitutes a treatment of choice in cases of acute salmonellosis due to multiresistant strains[10–13], whose prevalence is increasing[14,15].

In *Salmonella* spp., quinolone resistance has become attributed to point mutations in genes for DNA gyrase, whose complex with DNA is primary target of quinolones[7,8]. In addition to this mechanism of target modification that is strictly specific to quinolones, gram-negative bacteria can face the presence of toxic compounds, including antibiotics, by excluding them from cell. Changes in cell envelope, including loss of outer membrane porins or alterations of the lipopolysacharide, can be partially responsible for decreased susceptibility to a wide range of unrelated antibiotics[16,17]. Also some of the multidrug efflux pumps, which belong to several families, exhibit a low specificity and thus confer decreased susceptibility or even clinically significant resistance to several classes of antibiotics when they are overexpressed[18].

Therefore the aim of this work was to examine the effect of 2-nitrofluorene on induction of mutations leading to ciprofloxacin resistance in *Salmonella typhimurium.*

MATERIAL AND METHODS

Bacterial strain and chemicals: The bacterial strain used in this study, quinolone-susceptible *Salmonella typhimurium* TA 98 was received from the Collection of Microorganisms, Masaryk University, Brno (Czech Republic). 2-nitrofluorene and ciprofloxacin were purchased from Sigma-Aldrich (Germany).

Antimicrobial susceptibility testing: The MICs were determined by agar dilution method using agar plates containing serial twofold dilutions of drug, and by microsuspenssion assay in 96-well microplates. The MIC was defined as the lowest concentration of drug that completely inhibited visible growth of the bacteria after incubation for 18h at 37 °C.

Ames test: The test was performed using the classical plate incorporation method according Maron and Ames[19].

Determination of mutation frequency to antibiotic resistance: 0.1 ml of overnight culture (cultivation for 16 h at 37 °C, approximate cell density 2–5 × 10^8 cells/ml), 0.1 ml of a solution of 2NF and 0.5 ml of phosphate buffer of pH 7.4 were mixed and preincubated at 37 °C for 30 min. Then 0.7 ml of double strength nutrient broth was added and the culture were incubated 3 h at 37 °C

to allow a few cell divisions and protein expression to occur. The top agar supplemented with ciprofloxacin was added, test tubes were vortexed rigorously and the number of resistant revertants that emerged in each culture was determined by plating the entire culture on agar plates containing ciprofloxacin at concentration of 2 × MIC. Plates were incubated aerobically for 72 h at 37 °C.

Cytotoxicity assay: The number of viable cells was determined by plating an appropriate dilution of three cultures on nonselection medium. Plates were incubated aerobically for 24 h at 37°C.

Statistical evaluation of results: Experiments were repeated independently at least three times in five parallel estimation for each 2NF concentration and results were statistically evaluated using the Student's *t*-test. The frequency of resistant mutants (resistance index RI) was expressed as the mean number of resistant colonies divided by the total number of viable cells per culture.

RESULTS AND DISCUSSION

Isolation of ciprofloxacin-resistant strains

Spontaneous resistant mutants of *Salmonella typhimurium* appeared at frequencies ~9 × 10^{-8} after 72h of incubation on agar plates containing ciprofloxacin at 2 × MIC. The resistance index of cells that grew at the same conditions increased after previous exposure to 2NF to values ranging from 1×10^{-6} to 5×10^{-6} depending from the 2NF concentration. At the concentration of 10 μg/0.1 ml of 2NF we achieved 57 fold increase of RI. The relationship between the concentration of mutagen and resistance index is evident from Fig. 1. The number of revertants in the Ames test confirmed the mutagenic activity of 2-nitrofluorene in used concentrations.

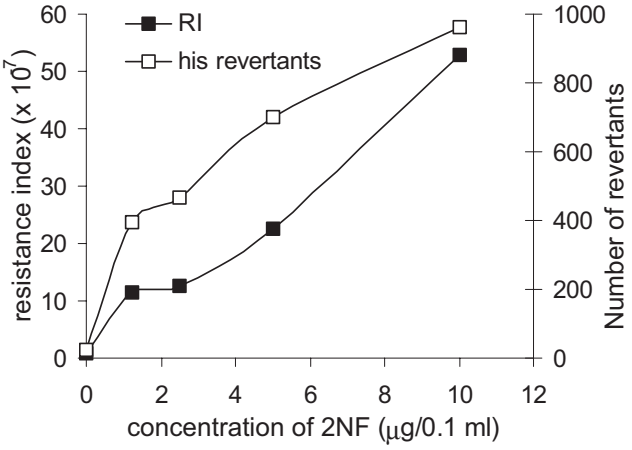
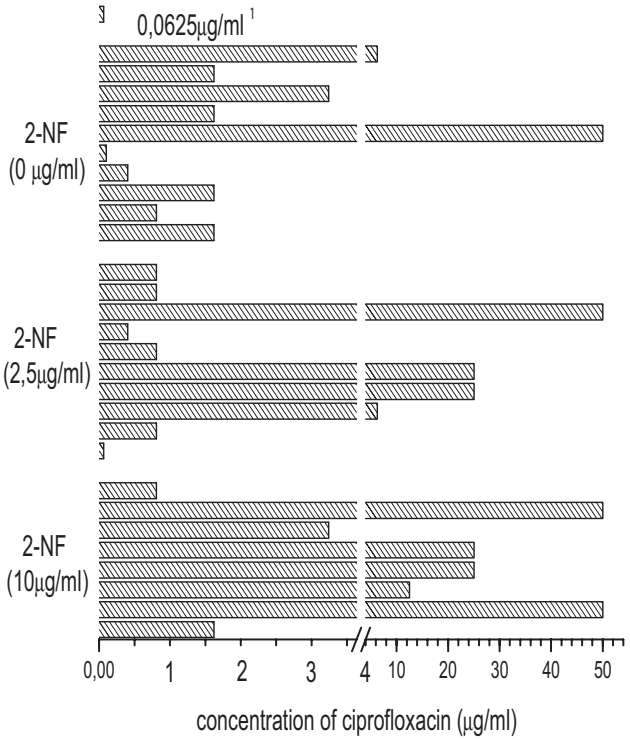


Fig. 1. Mutation frequency (resistance index RI) to ciprofloxacin resistance and the number of *his*+ revertants of *Salmonella typhimurium* induced by 2-nitrofluorene.



[1] selective concentration of ciprofloxacin

Fig. 2. The highest values of ciprofloxacin concentration which allowed the visible growth of isolated bacterial strains. Each column represents one bacterial culture isolated on selective plate as a consequence of spontaneous (0 μg/ml of 2-NF) or 2-NF–induced mutations. Strains growing in the concentrations of ciprofloxacin > 4 μg/ml (break) are considered to be resistant.

Sensitivity of isolated cultures to ciprofloxacin

Although the resistant mutants were isolated at concentration of ciprofloxacin of 0.06 μg/ml, they were resistant not only to selective concentration. From each culture which grew on selective plates after previous treatment with 2-NF, one mutant colony was picked and evaluated for ciprofloxacin MIC. It is evident from Fig. 2, that isolated cultures were capable to grow on the plates with higher concentrations of ciprofloxacin in some cases even at concentration of 50 μg/ml.

According to National Antimicrobial Susceptibility Program[20], ciprofloxacin susceptibility limit for *Salmonella typhimurium* is 4 μg/ml, and bacteria which grow in the presence of ciprofloxacin in these and higher concentration are considered to be resistant. When classifying isolated mutant strains according of this criterion, some of selected mutants are resistant and some have only decreased sensitivity to ciprofloxacin.

The development of antibiotic resistance among bacterial populations reflects the interplay of intrinsic genetic features of the strain and exogenous environmental factors[21]. Our results provide evidence that environmental mutagens may contribute to the development of bacterial antibiotic resistance.

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