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ORIGINAL ARTICLE

Nitrofurantoin, phenazopyridine, and the superoxide-response regulon *soxRS* of *Escherichia coli*

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Abstract Nitrofurantoin and phenazopyridine are two drugs commonly used against urinary tract infections. Both compounds exert oxidative damage in patients deficient in glucose-6-phosphate dehydrogenase. This study was done to assess the interactions of these drugs with the *soxRS* regulon of *Escherichia coli*, a superoxide-defense system (that includes a nitroreductase that yields the active metabolite of nitrofurantoin) involved in antibiotic multi-resistance. The effects of either nitrofurantoin or phenazopyridine, upon strains with different *soxRS* genotypes, were measured as minimum inhibitory concentrations (MICs) and growth curves. Also, the ability of these drugs to induce the expression of a *soxS'::lacZ* gene fusion was assessed. The effect of antibiotics in the presence of phenazopyridine, paraquat (a known *soxRS* inducer), or an efflux inhibitor, was measured using the disk diffusion method. A strain constitutively expressing the *soxRS* regulon was slightly more susceptible to nitrofurantoin, and more resistant to phenazopyridine, compared to wild-type and *soxRS*-deleted strains, during early treatment, but 24-h MICs were the same (8 mg/l nitrofurantoin, 1,000 mg/l phenazopyridine) for all strains. Both compounds were capable of inducing the expression of a *soxS'::lacZ* fusion, but less than paraquat. Subinhibitory concentrations of phenazopyridine increased the antimicrobial effect of ampicillin, chloramphenicol, tetracycline, and nitrofurantoin. The induction or constitutive expression of the *soxRS* regulon seems to be a disadvantage for *E. coli* during nitrofurantoin exposure; but might be an advantage during phenazopyridine exposure, indicating that the latter compound could act as a selective pressure for mutations related to virulence and antibiotic multi-resistance.

Keywords Antibiotic resistance · *Escherichia coli* · Nitrofurantoin · Phenazopyridine · *soxRS* · Urinary tract infection

Introduction

Several drugs can be used in the therapy of lower urinary tract infections (UTI). Of these, two are old and their precise mechanism of action is mostly unknown: the antimicrobial drug nitrofurantoin (NF, patented in 1952), and the urospecific analgesic phenazopyridine (PP, patented in 1927–1928). NF is used against lower UTI, and PP is often used along with an antibiotic, occasionally NF itself, also in the management of UTI, particularly in developing countries. Although it is commonly accepted that PP is devoid of antibacterial properties [1], NF is known to be a sort of prodrug, requiring an enzyme-mediated reaction to generate derivatives [2] that are involved in various kinds of damage to the bacterial cell. These notions are, however, controversial: one of the earliest reports on PP states a "bacteriostatic action against *Staphylococcus aureus* and *Escherichia coli*" at 600-mg doses [3]; and a paper on the mechanisms of action of NF reports one that "does not require the production of reactive NF metabolites by bacterial reductases" [4]. In addition to being used in urinary tract ailments, NF and PP have in common being compounds capable of inducing oxidative injury to erythrocytes,

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especially in individuals with glucose-6-phosphate dehydrogenase (G6PD) deficiency [5].

Our first objective was to assess the interactions of each of these compounds, with the response of *Escherichia coli* to superoxide stress governed by the *soxRS* genes. The products of these genes act sequentially: first, SoxR becomes a transcriptional activator of *soxS*, in the presence of superoxide stress (likely because of diminishing NADPH concentrations [6]); SoxS, in turn, activates the expression of defense and repair mechanisms that include a Mn-containing superoxide-dismutase, NADPH-replenishing G6PD, DNA-repairing endonuclease IV, and the xenobiotic efflux system AcrAB, among many others [7]. A second objective was to explore the potential paradox between NF action and the *soxRS* response: one of the two reductases, NfsA, that turn NF into an antimicrobially active compound [8], is under the control of *soxRS* [9]. However, at least two other *soxRS*-controlled genes (*acrAB*, mentioned above; and *micF*, encoding an antisense RNA that post-transcriptionally inhibits the expression of OmpF, hence diminishing the permeability of the outer membrane) mediate unspecific antibiotic resistance. Therefore, although the induction of the *soxRS* system (or its constitutive expression caused by mutations) could potentially increase the conversion of NF into an active compound, it could also provide the means for resisting its antimicrobial effects.

Materials and methods

Strains and media

Escherichia coli K12 strains with three different *soxRS* genotypes were used: GC4468, carrying wild-type (wt) *soxRS* genes; DJ901, a derivative of GC4468 but with the *soxRS* genes deleted (Δ*soxRS*); and JTG1052, carrying allele *soxR101* (*soxR*^{c}), that constitutively expresses *soxS*, hence the entire regulon [10]; and also, strain TN521, a derivative of DJ901 carrying a prophage with a wild-type *soxR* gene, and a *soxS'::lacZ* fusion; and TN531, the same as TN521 but without the *soxR* gene [11]. All these strains were a kind gift from B. Demple. Cells were grown on liquid or solid LB media [except for antibiotic susceptibility assays, performed on liquid or solid Mueller–Hinton media (MH; Fluka)].

Antibacterial activity of NF and PP

Minimum inhibitory concentrations (MIC) of NF (Sigma) and PP (3-phenylazo-2,6-diaminopyridine hydrochloride; Alfa Aesar) were assessed by twofold serial dilution on MH broth, incubated for 24 h at 35 °C without agitation: the NF series was 128, 64, 32, 16, 8, 4, 2, and 1 μg/ml; the PP series was 1,000, 500, 250, 125, 62.5, 31.3, 15.6, and 7.8 μg/ml. Additionally, cells were treated with subinhibitory concentrations of NF (5 μg/ml) or PP (100 μg/ml), in MH broth incubated at 35 °C, agitated at 200 rpm, sampling each hour, diluting and plating on LB agar; plates were incubated at 35 °C for 18 h, and colonies were counted.

Measurement of *soxS'::lacZ* induction

The activity of β-galactosidase was measured as described by Miller [12], after treating strains TN521 and TN531 with subinhibitory concentrations of NF or PP (paraquat, PQ, a redox-cycling, superoxide generating agent, and a known inducer of the *soxRS* regulon [7, 11], was used as positive control), for 30 min at 35 °C, agitated at 200 rpm. In experiments with PP, cells were first washed twice in ice-cold Z buffer, as PP interferes with the measuring of hydrolyzed ONPG at 420 nm.

Effect of PP or efflux-pump inhibitor upon the activity of NF and other antibiotics

The activity of ampicillin, chloramphenicol, tetracycline, and NF was measured using the disk diffusion method (Bauer-Kirby) on MH agar plates with or without PP (100 μg/ml), PQ (50 μM), or Phe-Arg-β-naphthylamide (PAβN, at 50 μM [13], a known inhibitor of xenobiotic efflux mediated by AcrAB, a member of the *soxRS* regulon [7]). Overnight cultures were diluted in fresh MH broth, incubated at 35 °C until OA_{600} of 0.5, pretreated with PP 100 μg/ml or PQ 50 μM for 30 min, and streaked on MH agar plates; antibiotic disks were applied, and plates were incubated at 35 °C for 18 h.

Results

MICs of NF and PP

The growth of all three strains with different *soxRS* genotypes was completely inhibited by NF at 8 μg/ml after a 24-h incubation. MIC of PP was 1,000 μg/ml for all three *soxRS* strains as well, although turbidity of JTG1052 was slightly higher at 500 μg/ml (not shown).

Growth curves

Hourly monitoring bacterial growth in the presence of subinhibitory concentrations of NF (5 μg/ml) or PP