Inhibition of Conjugal Transfer of R Plasmids by Nitrofurans

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Nifurzide is a nitrofurantoin with antibacterial activity. As nitrofurans have been reported to interact with DNA, we tested the ability of nifurzide to inhibit plasmid transfer. Inhibition of plasmid transfer between *Escherichia coli* strains was obtained for ten plasmids belonging to nine incompatibility groups. The same effect was observed when plasmid RP4 was harboured in six different members of the *Enterobacteriaceae*. Inhibition depended on the reduction of the –NO₂ group of nifurzide and was obtained with four other nitrofurantoin derivatives.

INTRODUCTION

Bacterial resistance to antibiotics mediated by plasmid genes is widespread. Some compounds, namely acridine derivatives, 'cure' or eliminate R plasmids (Watanabe & Fukasawa, 1961), while a few antibiotics interfere with maintenance or transfer of R plasmids, namely rifampicin (Fenwick & Curtiss, 1973*b*), nalidixic acid (Chabbert *et al.*, 1969; Fenwick & Curtiss, 1973*a*) and its derivatives (Nakamura *et al.*, 1976), clindamycin (Coats & Roeser, 1977), trimethoprim (Pinney & Smith, 1972) and nitrofurantoin (Shenderov, 1971). Most of these substances seem to exert their antibacterial effect by interfering, directly or indirectly, with DNA replication, and this is true for nitrofurantoin (Iida & Koike, 1977). Here we report that several nitrofurans inhibit plasmid transfer. A preliminary report has been published (Michel-Briand & Laporte, 1982).

METHODS

Bacterial strains. *Escherichia coli* K12 (J53) (*met-63, pro-22*) and UB1139 (*leu met thy nal*) were obtained respectively from N. Datta (Royal Postgraduate Medical School, Hammersmith Hospital, London, UK) and P. M. Bennett (Dept of Bacteriology, University Walk, Bristol, UK). *E. coli* AB1157 *thr-1 leu-6 thi-6 lacY1 galK2 ara-14 xyl-5 mtl-1 proA2 argE3 str-31 tsx-33 nfsA⁺B⁺* and its *nfsAB* mutant, NFR502, were provided by D. R. McCalla (Dept of Biochemistry, McMaster University, Hamilton, Ontario, Canada). *Salmonella typhimurium* LT2-M329 Trp[–], *Shigella sonnei* 7823, *Klebsiella pneumoniae* NCTC 234 and *Serratia marcescens* SM were obtained from N. Datta and *Proteus mirabilis* PM1 (*nad str*) from P. M. Bennett. *Pseudomonas aeruginosa* PAO8 (*met-28 ilv-202 str-1*) was the strain of Isaac & Holloway (1968) and *Pseudomonas aeruginosa* PAO2635 (*trp-54 rif-3 fon-101*) was from V. Stanisich (Dept of Microbiology, La Trobe University, Bundoora, Victoria, Australia).

Plasmids. R386 (Tc) (IncFI), R446b (Sm, Tc) (IncM), Sa (Sm, Km, Cm, Su) (IncW) and R6K (Cb, Sm) (IncX) were gifts from N. Datta; pIP24 (Tc) (IncFII) and R64 (Sm, Tc) (IncII) were obtained from P. Courvalin (Institut Pasteur, Paris, France); RGN238 (Ap, Sm, Tc, Cm, Su) (IncFI) was from M. Matthew (Glaxo Holdings Ltd, London, UK); N3 (Sm, Tc, Su) (IncN) was from E. L. Lederberg (Dept of Medical Microbiology, Stanford University, Stanford, Calif., USA); RP4 (Km, Nm, Tc, Ap) (IncP) was from P. M. Bennett; pYMB1 (Cb, Sm, Sp, Nm, Pm, Lv, Rm, Gm, Ss, Cm, Tc, Su) (IncPI) was from this laboratory (Michel-Briand *et al.*, 1981).

Media and antibiotics. A semi-solid medium [heart-brain infusion (Institut Pasteur Production) with 0.08% (w/v) agar] layered on a solid medium (Mueller–Hinton, Institut Pasteur Production) was used to enumerate viable bacteria. Antibiotics were incorporated as appropriate at the following concentrations: carbenicillin (Cb) 500 µg ml^{–1}; cloxacillin (Cl) 800 µg ml^{–1} (Beecham-Sevigné, Paris); nalidixic acid (Nal) 100 µg ml^{–1} (Winthrop,

Abbreviation: MITC, minimum inhibition of transfer concentration.

Clichy, France); rifampicin (Rf) 100 µg ml⁻¹ (Lepetit, Neuilly-sur-Seine, France); tetracycline (Tc) 40 and 100 µg ml⁻¹ (Pfizer, Orsay, France); chloramphenicol (Cm) 50 µg ml⁻¹ (Roussel, Paris); ampicillin (Ap) 100 µg ml⁻¹ (Bristol, Paris); novobiocin (No) (Theraplix, Paris). Suitable inhibitory concentrations of nifurzide (Anphar-Rolland, Chilly-Mazarin, France) were determined for each conjugation. The compound was dissolved in dimethylsulphoxide/water to give a final concentration of 1.2% (v/v) dimethylsulphoxide (DMSO) in the culture medium. Minimum inhibitory concentrations (MIC) were determined according to Thrupp (1980). Other nitrofurans used were: methylfuradoin (1-[(5-methyl-2-furanyl)methylene]amino)-2,4-imidazolidinedione) and hydrofuradoin (1-[(2-furanyl)methylene]amino)-2,4-imidazolidinedione) (generously provided by M. Dershwitz, Dept of Pharmacology, Northwestern University, Medical and Dental Schools, Chicago, USA); and furazolidone and nitrofurantoin, methylmercadone and hydroxymethylnitrofurantoin (gifts from Oberval, Fumouze and Arsac laboratories respectively).

Bacterial matings. Bacteria were grown in heart-brain infusion broth at 37 °C in a rotary shaker for 3 h; matings were performed between 10⁸ donor bacteria ml⁻¹, harbouring the plasmid under test, in the exponential growth phase, and 10⁹ recipient bacteria ml⁻¹ in a final volume of 4.5 ml heart-brain infusion broth. Donor and recipient viable counts were made for each mating. Most matings were between *E. coli* J53 donor, and *E. coli* UB1139 recipient strains. Mating mixtures were incubated in test tubes in a 37 °C water bath, without shaking.

In preliminary experiments mating mixtures were incubated for 3 h with a range of nifurzide concentrations (30, 45, 60, 75, 90, 100, 120, 140, 180, 220 and 440 µM). The lowest concentration that inhibited the appearance of transconjugants and gave a less than 10-fold decrease of donor or recipient bacteria at 3 h was taken as the 'minimum inhibition of transfer concentration' (MITC).

Nifurzide at the MITC was then used in kinetic studies of plasmid conjugation. Transconjugants were assayed after 5, 10, 15, 20, 25, 30, 40, 60 min and 2 h. One control mating without nifurzide, and another with the solvent DMSO (1.2% v/v) alone were always done in parallel. After the appropriate incubation the mating mixtures were shaken vigorously for 15 s, and then transconjugants were selected by inoculating a dilution of the mating mixture onto 5 ml semi-solid medium containing appropriate selective agents (e.g. Cb + Nal for J53(RP4) × UB1139). This was layered onto solid medium containing the same antibiotics. Transconjugant colonies were counted after 24 h incubation at 37 °C. Donor and recipient counts were obtained by plating samples of the mating mixture on an appropriate selective medium.

The transfer frequency was defined as the ratio of transconjugants to donors in a particular volume of mating mixture.

Matings between *E. coli* J53(RP4) and *E. coli* UB1139 were done more than 20 times. In all experiments a decrease in transfer frequency of 10³ to 10⁴ was always found. Experiments with other donor strains were done no more than twice, but always with a control without nifurzide. Nine different concentrations were tested for each mating.

RESULTS AND DISCUSSION

Kinetics of appearance of transconjugants between E. coli J53(RP4) and UB1139

Conjugation without nifurzide. Under our experimental conditions, transconjugants were detected as soon as 5 min after the beginning of the mating for RP4. The number of transconjugants increased for 2 h (at this time, the transfer frequency was 10⁻³). In a medium containing DMSO (1.2%) the kinetics of plasmid transfer were unaltered.

Conjugation in the presence of nifurzide at the MITC. The MITC (60 µM, i.e. 20 µg ml⁻¹) was higher than the subinhibitory concentration obtained during the determination of the MIC (respectively 2.3 and 7.1 µM, i.e. 0.8 and 2.4 µg ml⁻¹, after 7 h incubation for donor and recipient respectively) probably because in this determination only one bacterial type was tested and at a much lower cell density (10⁶ bacteria ml⁻¹) than that used during the matings. No transconjugants were obtained from matings of up to 3 h when nifurzide was added at the beginning of the mating. Furthermore, the addition of nifurzide (to 60 µM) 20, 40, 60, 90 min after mixing donor and recipient cultures prevented further increase in the number of transconjugants obtained.

Similar results were obtained with nine other plasmids (R386, R446b, Sa, R6K, pIP24, R64, RGN238, N3, pYMB1) belonging to eight incompatibility groups, and also with four other nitrofurans derivatives (furazolidone, nitrofurantoin, hydroxymethylnitrofurantoin and methylmercadone).

Table 1. Matings between different donor bacteria harbouring RP4 and *E. coli* UB1139

Donor strain	Transfer frequency*		MITC† (μM)
	Without nifurzide	With nifurzide	
<i>Escherichia coli</i> J53(RP4)	2×10^{-3}	$< 1 \times 10^{-7}$	60
<i>Salmonella typhimurium</i> LT2(RP4)	2×10^{-4}	$< 5 \times 10^{-7}$	80
<i>Shigella sonnei</i> 7823(RP4)	1.3×10^{-4}	$< 1 \times 10^{-7}$	60
<i>Proteus mirabilis</i> PM1(RP4)	2×10^{-4}	$< 6 \times 10^{-8}$	60
<i>Klebsiella pneumoniae</i> 234(RP4)	1.4×10^{-5}	$< 5 \times 10^{-8}$	60
<i>Serratia marcescens</i> SM(RP4)	5×10^{-4}	$< 1 \times 10^{-7}$	45

* Transfer frequency at 3 h after the beginning of the mating, calculated as (number of transconjugants at 3 h)/(number of donors at the beginning of the mating).

† The lowest concentration of nifurzide which inhibited plasmid transfer (as described in Methods) and which was used in the matings.

Conjugation between different Enterobacteriaceae harbouring RP4 and *E. coli* UB1139

Nifurzide at the MITC, determined as described previously, inhibited plasmid transfer from all six strains of *Enterobacteriaceae* studied (Table 1). In contrast, no inhibition of transfer was observed between two *Pseudomonas aeruginosa* strains (PAO8 and PAO2635) intrinsically resistant to nifurzide (MIC > 440 μM).

Nature of chemical group involved in the plasmid transfer inhibition

Three very closely related compounds, nitrofurantoin (an antibacterial compound), hydrofurantoin and methylfurantoin (both devoid of any antibacterial activity, and where the $-\text{NO}_2$ group of nitrofurantoin is replaced by a hydroxyl and a methyl group respectively; Dershwitz & Novak, 1980) were tested for their effect on RP4 transfer. Using the same molar concentration of each compound (100 μM), a decrease in the yield of transconjugants was seen only with nitrofurantoin. Moreover the $-\text{NO}_2$ group of this compound must be in the reduced state for it to inhibit transfer, for inhibition occurred if the donor strain (*E. coli* AB1157) contained a high level of reductase I but was almost absent with a donor strain (*E. coli* NFR502) lacking reductase I.

The lack of progeny from conjugations in the presence of nifurzide was not due to plasmid curing, since when *E. coli* J53(RP4) was exposed to subinhibitory concentrations of this compound for 18 h at 37 °C, no loss of plasmid was observed (over 8000 colonies tested). The same results were found at the MITC (exposure for 3 h). Furthermore, nifurzide did not seem to act by affecting the bacterial surface or sex pili in some way, because there was no alteration of attachment of bacteriophages 434, TU1A and K10 (specific for OmpC, OmpF and LamB outer membrane porins respectively), nor of RP4 bacteriophage (specific for RP4 plasmid pili) (results not shown). In addition, because conjugation between two *Pseudomonas aeruginosa* strains resistant to nitrofurantoin by impermeability was not inhibited, we conclude that nifurzide probably enters the cell to exert its action.

Lastly, *gyrA* and *gyrB* mutations failed to confer resistance to nifurzide (results not shown).

In conclusion, nifurzide has been shown to inhibit the transfer of plasmids belonging to different incompatibility groups, between different members of the *Enterobacteriaceae*. Inhibition was due to the reduced form of the compound. However, it was not possible to distinguish between an inhibition due to a specific effect on plasmid replication and a less specific alteration of the metabolic activity of the host bacteria. Nonetheless, it is interesting to speculate from the experiments of Wagner *et al.* (1977), concerning the induction of β -galactosidase in the presence of nitrofurantoin, that a new RNA involved in plasmid replication is not translated while a nitrofurantoin derivative is present.

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