

ANTIMICROBIAL RESISTANCE

Plasmid-mediated rifampicin resistance in *Pseudomonas fluorescens*

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Rifampicin is an antibiotic mostly used to treat tuberculosis and leprosy, and, occasionally, other diseases. Resistance is due to alterations in membrane permeability or to mutation in the *rpoB* gene coding for mRNA polymerase. Both these mechanisms originate via chromosomal mutation. However, a rifampicin-resistant *Pseudomonas fluorescens* strain harboured a multiresistance plasmid which transferred rifampicin resistance when transformed into *P. putida* or *Escherichia coli*. Rifampicin readily diffused into the sensitive cells of the *E. coli* and *P. putida* recipients, but the transformants with the plasmid, pSCL were resistant to the drug and did not accumulate it. Potassium cyanide restored the diffusion of rifampicin into the resistant cells, indicating that an efflux pump was involved in the resistance mechanism. The resistance of the transformants and the wild strain was also abolished in sphaeroplasts generated by EDTA lysozyme treatment. Analysis of membrane proteins by SDS-PAGE revealed the presence of two new proteins in the plasmid-containing cells of *E. coli*, *P. putida* and *P. fluorescens* and not in the plasmid-free cells. These may be involved in the efflux of rifampicin.

Introduction

Bacterial resistance to antibiotics is a serious clinical problem, because it increases the morbidity, mortality and costs associated with disease [1]. Resistance arises by three major mechanisms: (i) drug inactivation, (ii) reduced drug accumulation and (iii) target alteration [2]. Antibiotic resistance can be specified by chromosomal or plasmid-encoded genes. Plasmid-mediated resistance is the more serious, as plasmids allow horizontal transfer, disseminating resistance genes to other bacteria.

Rifampicin is effective against a wide spectrum of pathogens and is a valuable first-line drug for tuberculosis and leprosy [3]. It inhibits bacterial RNA synthesis by binding to the β -subunit of the DNA-dependent RNA polymerase [4]. Resistance mostly results from amino acid substitution, or small insertions and deletions, within three short highly conserved regions of the β -subunit of the RNA polymerase. These modifications decrease the affinity of the antibiotic for its target site [5]. Hui *et al.* [6] suggested that membrane impermeability might also mediate rifampicin resistance in some strains of *Mycobacterium intracellulare*, where the RNA poly-

merase itself remained susceptible. All these mechanisms reflect chromosomal changes [7], but the present study reports plasmid-mediated rifampicin resistance in *Pseudomonas fluorescens*.

Materials and methods

Bacterial strains

P. fluorescens CAS 102 was isolated from polluted soil. It harboured a single large 140-kb plasmid, pSCL, encoding resistance to multiple antibiotics [8]. *E. coli* HB 101 (*supE44*, *ara14*, *galK2*, *lacY1*, *recA13*, *proA2*, *mtl13*, *xy15*) and *P. putida* DSM 2112 (Str⁺, Trp⁻, plasmid-free) were from the Institute of Microbial Technology, Chandigarh, India. Cultures were grown in Luria Broth (tryptone 1%, yeast extract 0.5%, NaCl 0.5%) at 30°C.

Plasmid isolation and transformation techniques

Plasmid DNA from *P. fluorescens* CAS 102 was isolated and resolved in agarose 0.6% gel electrophoresis at 50 V with TAE electrode buffer as described previously [8]. The DNA was visualised by staining the gel with ethidium bromide (1 mg/L) for 15 min and was photographed under short-wave UV light. Transformation of logarithmic-phase *E. coli* HB 101 cells was performed by the calcium chloride procedure of

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Maniatis *et al.* [9], and plasmid-free *P. putida* cells were transformed as described by Berry and Kropinsky [10]. Transformants were selected in LB medium with tetracycline 25 mg/L, chloramphenicol 25 mg/L, gentamicin 25 mg/L or kanamycin 25 mg/L. Transformants that could grow in the presence of all these antibiotics were then screened for resistance to rifampicin. Transformation was further confirmed by isolating the plasmid DNA from the transformants.

Rifampicin uptake

Rifampicin (Sigma) is a coloured substance with an absorbance peak at 473 nm. The procedure of Nikaido *et al.* [11] was adopted, with modifications, to study its uptake by bacteria. Cells were grown overnight in Luria broth and harvested by centrifugation. They were washed once and resuspended in 0.1 M phosphate buffer, pH 7.0, at an OD₆₀₀ of 0.5. To 1 ml of cell suspension, 50 µg of rifampicin (from a 1 mg/ml stock solution in methanol) was added, and the decrease in OD₄₇₃ was recorded continuously with a Philips PU 8740 UV/Vis Scanning Spectrophotometer. In some tests potassium cyanide was added to the cell suspension to a final concentration of 0.1 M.

Formation of sphaeroplasts

Sphaeroplasts were prepared by the procedure of Nikaido [12], with slight modifications. Organisms were grown overnight in Luria broth and harvested by centrifugation at 6000 g for 10 min at 4°C. The cells, at a density of 10 mg wet weight/mL, were suspended in 45 ml of ice-cold 0.75 M sucrose in 0.01 M Tris-HCl buffer, pH 7.5. After mixing, 5 mL of sucrose in Tris buffer containing lysozyme (Sigma) 10 mg were added. This was followed by incubation for 2 min at 0°C, then the suspension was diluted by adding 100 ml of ice-cold water over 5 min, with constant stirring. The suspension was then centrifuged at 3000 g for 10 min at 4°C. The pellet was carefully resuspended in 1.5 ml of 0.25 M sucrose in 0.01 M Tris-HCl buffer, pH 7.5 and equilibrated to 14°C in a water-bath, after which 0.3 ml of 0.25 M sucrose containing 0.05 M EDTA was added. The formation of sphaeroplasts was confirmed by microscopy, then rifampicin 50 mg/L was added, and the course of uptake was recorded as for intact cells (above).

Analysis of outer-membrane proteins

Outer membrane proteins (OMPs) were prepared as described by Cohen *et al.* [13], and resolved by SDS-PAGE [14] in gels containing acrylamide 10%. Proteins were visualised by staining with silver nitrate [15].

Results

P. fluorescens CAS 102 was resistant to rifampicin and to many antibiotics, dyes and heavy metals [8]. The

rifampicin MIC, initially around 20 mg/L, increased when the antibiotic concentration was incrementally raised over time. The organism harboured a single 140-kb, plasmid designated pSCL, which was extremely recalcitrant to curing. Hence, to understand the basis of rifampicin resistance, two plasmid-free bacterial strains, i.e., *E. coli* HB 101 and *P. putida* DSM 2112, were transformed with pSCL. These recipients initially were sensitive to rifampicin (MIC < 5 mg/L), but became resistant after transformation. They, like *P. fluorescens* CAS 102, also became resistant to tetracycline, gentamicin, kanamycin and chloramphenicol. Isolation of plasmid DNA from the transformants confirmed the presence of pSCL. Moreover, they, unlike *P. fluorescens* CAS 102, were amenable to curing, losing the plasmid upon repeated subculture in nutrient broth. After curing, these cultures became sensitive to rifampicin, tetracycline, gentamicin, kanamycin and chloramphenicol.

To determine the mechanism of resistance, the plasmid-free and transformed *E. coli* and *P. putida*, cells and *P. fluorescens* CAS 102 were assayed for rifampicin uptake (Fig. 1). The antibiotic readily diffused into the plasmid-free *E. coli*, and *P. putida* cells, as indicated by the decrease in OD₄₇₃, but there was no uptake by the transformant cells nor by *P.*

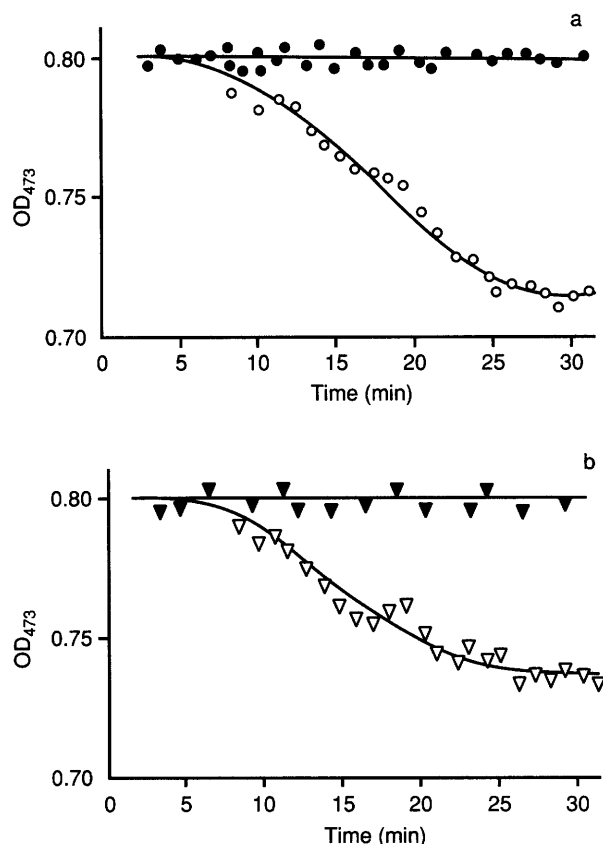


Fig. 1. (a) Rifampicin uptake by the plasmid-free cells of *E. coli* (○) and their transformants (●); (b) rifampicin uptake by the plasmid-free *P. putida* cells (▽) and their transformants (▼).

fluorescens CAS 102. To confirm that the decrease in OD_{473} was due to permeation of the drug, sensitive cells of *E. coli* and *P. putida* were centrifuged after incubation for 30 min with rifampicin 50 mg/L, washed in phosphate buffer and immediately sonicated. Debris was removed by centrifugation (6000 g at 4°C for 10 min). The supernates of the *E. coli* and *P. putida* sonicates contained 4.2 and 3.5 μg of rifampicin/mg dry cells, respectively. These values did not exactly match the original decline in absorbance, and a proportion of antibiotic may have been lost during processing. When transformed cells of *E. coli* and *P. putida* strains were treated similarly the sonicates contained negligible quantities of rifampicin.

When suspensions of *P. fluorescens* CAS 102 and transformants of *E. coli* and *P. putida* were incubated with potassium cyanide, as an energy uncoupler, rifampicin readily entered the cells (Fig. 2). This result confirmed that resistance was due to efflux rather than simple exclusion. When plasmid-free cells of *E. coli* and *P. putida* were incubated with potassium cyanide, reduction in the OD_{473} was observed, indicating that the drug entered the cells by diffusion.

The OMPs of the plasmid-free and transformed *E. coli* and *P. putida* cells, and the wild strain *P. fluorescens* were examined by SDS-PAGE. Changes were evident in transformants, with the appearance of two new proteins, which were not present in the plasmid-free strains (Fig. 3). These were designated Rir proteins (rifampicin-resistance proteins) and were produced irrespective of the presence or absence of rifampicin. The presence of the plasmid also repressed expression of two c. 29-kDa proteins in the *P. putida* transformant.

To confirm that resistance was solely due to efflux, resistant cells were converted to sphaeroplasts by lysozyme-EDTA treatment. Once the outer membrane

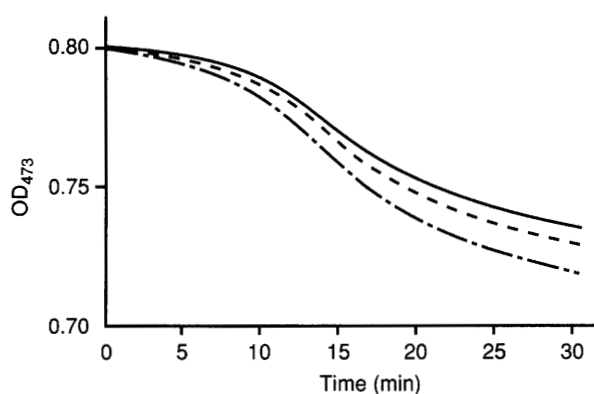


Fig. 2. Rifampicin uptake by *P. fluorescens* CAS 102 (—) and the resistant transformants of *E. coli* (- - -) and *P. putida* (- · -) in the presence of potassium cyanide.

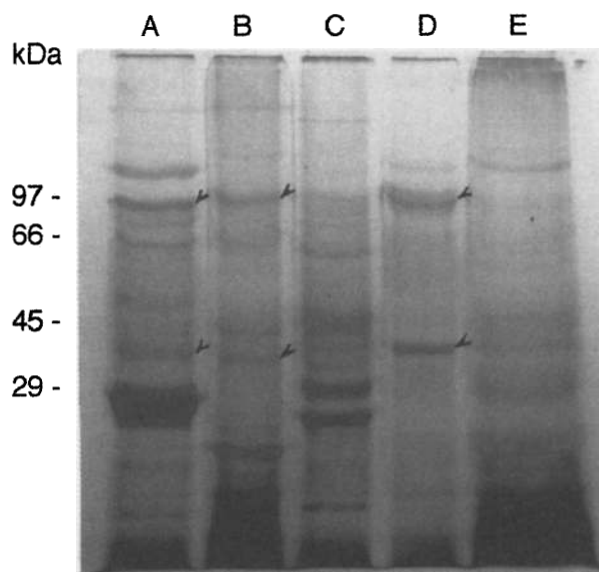


Fig. 3. SDS-PAGE of membrane proteins of *P. fluorescens* CAS 102 (lane A), *P. putida* transformed with pSCL (B), plasmid-free *P. putida* (C), *E. coli* HB101 with pSCL (D), plasmid-free *E. coli* HB101 (E). Arrows indicate the Rir proteins. Numbers on the left denote mol. wts.

barrier was removed by this process, it was observed that the rifampicin readily diffused into the cells. However, the rate of uptake by the sphaeroplasts of both *E. coli* and *P. putida* transformants was slightly less than by the intact plasmid-free sensitive cells. Uptake was further confirmed by release of diffused rifampicin from the sphaeroplasts of the transformants: sonicates of the *E. coli* and *P. putida* sphaeroplasts contained 3.1 and 2.2 μg of rifampicin/mg dry mass, respectively. Uptake of 2.1 μg of rifampicin/mg dry cells was also observed for the sphaeroplasts of *P. fluorescens* CAS 102.

Discussion

Rifampicin is a hydrophobic antibiotic and enters bacteria by dissolving into the hydrocarbon interior of the outer membrane and then diffusing into the aqueous phase of the periplasm. Finally, it diffuses across the cytoplasmic membrane [12]. Resistance may arise by alteration in the outer membrane structure, as reported for a mutant of *Neisseria meningitidis*, where a significant decrease in the amount of C₁₆ outer membrane lipids appeared to be responsible [16]. Resistance may also arise by a single point mutation in the short central region of the *rpoB* gene, which codes for RNA polymerase [4]. However, in the present case, the results show that the resistance was due to plasmid-encoded OMPs.

Association between the production of novel OMPs and resistance to antimicrobial agents is well established. For example, *P. aeruginosa* with *nfxC* and

nfxB mutations [17, 18] show decreased susceptibility to several antimicrobial agents and concomitant production of 54- and 50-kDa OMPs, respectively. Poole *et al.* [19] reported that decreased drug accumulation by these mutants was not due to reduced outer membrane permeability, as originally suggested, but to efflux. It is now agreed that even the most effective permeability barrier could not effectively exclude small molecules, unless a second factor, in the form of drug inactivation or efflux is present [20]. The present study suggests participation of an efflux pump in rifampicin resistance, as addition of potassium cyanide would de-energise such a pump, leading to the diffusion of the antibiotic into the resistant cells—as was seen.

Unlike the mammalian multi-drug efflux pump, P-glycoprotein, which is ATP-dependent, most bacterial multidrug efflux pumps are driven by the proton motive force (PMF) of the transmembrane electrochemical gradient. The fact that cyanide abolished efflux in the present case implies that the rifampicin efflux pump reported here could belong to this category. PMF-dependent efflux pump systems may comprise OMPs as small as 15 kDa belonging to the *smr* family [21] or they may comprise multicomponent efflux machinery which includes proteins belonging to the major facilitator superfamily (MFS) or the resistance/nodulation/cell division (RND) family [22]. It is not clear whether the two membrane proteins reported here confer resistance only to rifampicin by selective efflux, as, e.g., with tetracycline transporters, or against a wide range of structurally dissimilar compounds as with some of the *P. aeruginosa* systems [19]. Plasmid pSCL codes for resistance to a large number of antibiotics, heavy metals and dyes and further biochemical and genetic analysis will reveal more about its function and nature.

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