R PLASMID-BORNE TRANSFERABLE MULTIPLE ANTIBIOTIC RESISTANCE IN A CLINICAL ISOLATE OF PROTEUS SP IN PENINSULAR MALAYSIA

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A clinical isolate of Proteus sp., resistant to ampicillin, carbenicillin, cephaloridine, chloramphenicol, cotrimoxazole, gentamicin, kanamycin and tetracycline, was examined for the presence of conjugative R plasmids. Results from conjugation, agarose gel electrophoresis and transformation experiments showed that it harboured a large self-transmissible R plasmid which coded for all the resistance traits.

INTRODUCTION

The introduction of antimicrobial therapy has been a great advance in the treatment of infectious diseases. However, in parallel with the extensive and intensive use of antibiotics in humans and animals, there is an increase in the emergence of antibiotic resistant strains of bacteria throughout the world (1, 2). In most of these bacteria, the genetic determinants for antibiotic resistance are located on stable, extrachromosomal, self-replicating DNA elements, called R plasmids, many of which can be transferred by conjugation from bacterium to bacterium even between different bacterial species (3, 4).

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In Malaysia, multiple antibiotic resistant bacterial strains have been isolated from clinical specimens in hospitals, and some of them have been shown to transfer part or all of their resistances (5 – 7). However, no attempt has been made to demonstrate physically the presence of R plasmids in these bacterial strains. In view of this, we chose to study a clinical isolate of *Proteus* sp. that has been found to be resistant to ampicillin (Ac), carbenicillin (Cb), cephaloridine (Ce), chloramphenicol (Cm), cotrimoxazole (Ct), gentamicin (Gm), kanamycin (Km) and tetracycline (Tc), but sensitive to nalidixic acid (Nx). We report here the genetic evidence which shows that all the antibiotic resistance traits in the *Proteus* strain were mediated by a large self-transmissible R plasmid.

MATERIALS AND METHODS

Bacterial strains: The *Proteus* isolate of known antibiogram was obtained from the Bacteriology Division, Institute for Medical Research, Kuala Lumpur, Malaysia. It was isolated from the urine of a patient.

Escherichia coli CSH56 (F^- *ara* △ (*lac pro*) *sup*D *nal*A *thi*) (8), sensitive to all antibiotics tested except Nx, was used as the recipient in mating experiment. *E. coli* JA221 (*rec*A1 *hsd*M^+ *hsd*R^- △ *trp*E5 *leu*B6 *lac*Y) (9), a plasmidless antibiotic sensitive strain, was used as the recipient in transformation experiment.

Media: LB and M9 media were prepared as described previously (7).

Conjugation procedure: The *Proteus* stain was mated with *E. coli* CSH56 to verify if the resistance traits were transferable, and quantitative bacterial mating was performed by a plate mating method as described previously (7). Transconjugants were selected on LB agar plates containing Nx (100 μg/ml) and Ac (100 μg/ml). Controls containing the donor and the recipient alone were treated in exactly the same way as the mixed mating. Neither the donor *Proteus* strain nor the recipient *E. coli* grew on the selective medium.

Analysis of transconjugants: To examine the acquisition of unselected resistance traits, single transconjugant colonies growing on the selective medium were purified and toothpicked onto different LB agar plates, each containing one of the following antibiotics to which the donor strain was resistant: Cb (100 μg/ml), Ce (100 μg/ml), Gm (10 μg/ml), Km (40 μg/ml) or Tc (20 μg/ml). The donor and recipient cells were also inoculated on the same antibiotic plates as the transconjugants to act as controls.

Transfer frequency is expressed as the number of transconjugants per ml of the mating mixture divided by the number of donor cells per ml of the same mating mixture.

Isolation of plasmid and agarose gel electrophoresis: The rapid extraction method of Kado and Liu (10) was used to isolate plasmid DNA from the *Proteus* donor and the *E. coli* transconjugants. Horizontal agarose gel electrophoresis for the detection of plasmid DNA was performed according to Meyers *et al.* (11). At the end of the electrophoretic run, gels were stained with ethidium bromide (5 μg/ml) and visualised and photographed on a 302 nm UV transilluminator (Model TM 36, UV Products, Inc.), using a Polaroid MP-4 Land camera system fitted with a yellow filter and Polaroid type 665 black-and-white Land films.

Transformation: Plasmid DNA extracted from one of the *E. coli* transconjugants by the method of Kado and Liu (10) was first dialysed against TE buffer (10 mM Tris-HC1, 1 mM EDTA, pH 7.5). It was then used to transform *E. coli* JA221 to Ac resistance and Km resistance, respectively, following the method described by Dagert and Ehrlich (12).

Analysis of transformants: Transformant colonies growing on the Ac and Km selective plates were purified and toothpicked onto different LB plates, each containing one of the following antibiotics to which the donor strain was resistant: Ac (100 μg/ml), Ce (100 μg/ml), Cm (25 μg/ml), Km (40 μg/ml) or Tc (20 μg/ml). To screen for the acquisition of Ct resistance, transformant colonies were toothpicked onto M9 minimal plates supplemented with glucose, leucine, tryptophan and Ct (containing 16 μg/ml trimethoprim and 80 μg/ml sulphamethoxazole). *E. coli* JA221 cells were also inoculated on the same antibiotic plates as the transformants to act as control.

Antibiotics: Antibiotics added to agar or broth were from the following sources: ampicillin sodium (Penbritin; Beecham Research Laboratories, England), carbenicillin sodium (Pyopen; Beecham), cephaloridine (Sigma Chemical Co., U.S.A.), chloramphenicol (Sigma), cotrimoxazole (Bactrim; SA F. Hoffmann-La Roche & Co. Ltd., U.S.A.), gentamicin sulphate (Sigma), kanamycin sulphate (Sigma), nalidixic acid (Sigma) and tetracycline HC1 (Sigma).

RESULTS

Transfer of Ac resistance by conjugation to *E. coli*: Ac resistance was transferred from the *Proteus* donor to *E. coli* CSH56 recipient by conjugation at a frequency of 8.0×10^{-4}

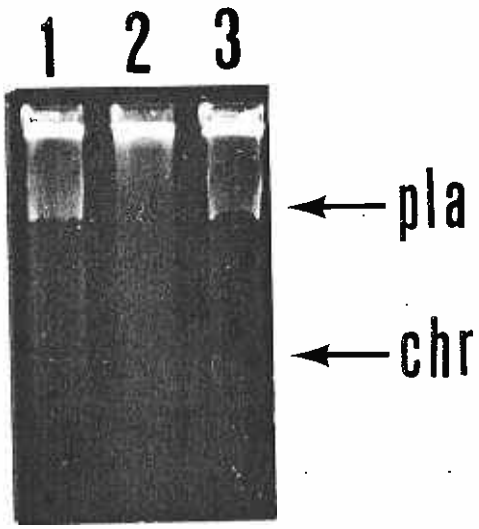

Figure 1. Agarose (0.5%) gel electrophoresis of DNA extracted from *Proteus* donor (1), *E. coli* CSH56 recipient (2), and *E. coli* CSH56 transconjugant (3). pla = ccc plasmid DNA; chr = chromosomal DNA.

Coinheritance of unselected resistance traits: Fifty *E coli* transconjugant colonies growing on the selective agar plates were purified and toothpicked onto media containing antibiotics to determine the nature of other antibiotic resistance traits that had been cotransferred during conjugation. All the transconjugants were found to have acquired resistance to Cb, Ce, Gm, Km and Tc. Resistance to Cm and Ct was not tested.

Analysis of plasmid profiles: The result of the mating experiment showing *en bloc* transfer of unselected resistance traits suggests that the donor *Proteus* strain harboured a conjugative R plasmid.

Fig. 1 shows that after electrophoresis a slow-migrating covalently closed circular (ccc) DNA band was detected in both the *Proteus* donor and the *E. coli* transconjugant, but was absent in the *E. coli* CSH56 recipient. This band shows the presence of a high molecular weight plasmid DNA in both the donor and the transconjugant.

Transformation: After dialysis against TE buffer, 25 μl of the plasmid DNA extracted from one of the *E coli* transconjugants was used to transform *E. coli* JA221. Both Ac resistant and Km resistant transformants were obtained, although at low frequencies (Table 1).

Coinheritance of unselected resistance traits: All transformant colonies were purified and toothpicked onto media containing antibiotics to determine the nature of other antibiotic resistance traits that had been simultaneously transferred by transformation. Table 1 shows that all the transformants were resistant to Ac, Ce, Cm, Ct, Km and Tc, regardless of whether initial selection was for Ac or Km resistance. Resistance to Cb and Gm was not tested.

DISCUSSION

Multidrug resistant *Proteus* spp. have been reported in the past several years (13 – 20). However, except for the strains reported by Traub *et al.* (13), Edwards *et al.* (16), Shafi and Datta (17), and Yoshikawa *et al.* (20), most of these *Proteus* strains do not show the magnitude of resistance reported here. This resistance to a wide range of antibiotics may be due to the indiscriminate use of antibiotics in Malaysia.

Genetic evidence from conjugation, agarose gel electrophoresis and transformation experiments shows that our multiresistant *Proteus* strain harboured a large conjugative R plasmid, which conferred resistance to all the eight antibiotics tested. This is the first direct evidence of a self-transferable multiresistance R plasmid in a clinical isolate of *Proteus* sp. in Malaysia. Our strain is different from those previously reported strains (13, 16, 17, 20) that have as large a number of resistances as encountered in our study, because they either fail to transfer their resistances or transfer only a part of their resistances by conjugation.

The discovery of a clinical *Proteus* isolate, harbouring a self-transmissible multiresistance R plasmid, is of concern to public health and therapeutic treatments. *Proteus* spp., particularly *P. rettgeri* and *P. mirabilis*, are known nosocomial pathogens causing urinary tract infection in compromised patients(13, 16 – 21). Furthermore, multiresistant bacterial strains that already carry conjugative R plasmids form a reservoir from which initially antibiotic sensitive pathogens may acquire drug resistances (4, 22, 23). In view of the potential hazard, it is therefore imperative that further investigations be conducted to look into the extent of plasmid-mediated transferable antibiotic resistances in *Proteus* and other clinical bacterial species in Malaysia.

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Table 1

Transformation of E. coli JA221 with plasmid DNA extracted from one of the E. coli transconjugants

Selective antibiotic	Number of transformant colonies	Number of transformants growing when toothpicked onto plates with Ac	Ce	Cm	Ct	Km	Tc	Resistance pattern transferred
Ac	5	5	5	5	5	5	5	AcCeCmCtKmTc
Km	10	10	10	10	10	10	10	AcCeCmCtKmTc

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