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# CHARACTERIZATION OF SOME R-PLASMIDS OF GRAM NEGATIVE BACTERIA

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## ABSTRACT

Six R-plasmids of Gram negative bacteria isolated from poultry in Karachi region designated as pFK-2, pFK-6, pFK-7, pFK-8a, pFK-13 and pFK-20 were studied for various genetic properties such as fi′ character, phage restriction and phage propagation in order to characterize them. Regarding their fi character, two (pFK-2 and pFK-7) were found to fi′ and four (pFK-6, pFK-8a, pFK-13 and pFK-20) were found to be $fi^+$. None of the R plasmids tested neither restrcited phages T6, Plkc, Mucts, ØX174 and λ of *E. coli* and P22 of Salmonella nor propagated the phages MS2, M13, f2, If1 and IKe. Frequency of transmission between *E. coli* strains of these R plasmids was also calculated and was found to be quite high during HFT state.

**Key words:** R plamid, bacteria, poultry, genetic properties

## INTRODUCTION

R plasmids are genetic determinants conferring resistance to a number of drugs or antibiotics (Clowes, 1973; Cohen and Miller, 1970; Harada *et al.*, 1960; Meynell *et al.*, 1968; Rownd *et al.*, 1966; Watanabe, 1967). Chemically, R plasmids consist of deoxyribonucleic acid (DNA) like the bacterial chromosome, although they behave independently in biophysical and genetic experiments (Clowes, 1972; Cohen *et al*., 1971). R plasmid is thus an independent linkage group, composed of genes determining drug resistance associated with others conferring the ability to conjugate and to transfer the plasmid to a new host. The later have been collectively referred to as the RTF (Resistance Transfer Factor), sex factor or tra region (Novick *et al.*, 1976). Some R plasmids are non-transmissible, but the majority of them are transmissible (Ansari and Khatoon, 1994). As mentioned above the portion of a transmissible R plasmid, which contains genes involved in conjugation, is resistance transfer factor (RTF), genes coding for antibiotic resistance are called resistance determinants. The two parts of the plasmid (i.e. the RTF and resistance determinants) can separate and behave as autonomous plasmids. A number of resistance determinants may become linked to one another and to an RTF, so that resistance to many antibiotics may be carried on a single plasmid (multiple drug resistance) (Cohen and Miller, 1970). Recombination between an R plasmid and the bacterial chromosome is rare, probably due to the absence of homology between them.

R plasmids are of two types, $fi^+$ and $fi^-$ (fertility inhibition positive and fertility inhibition negative) (Bannister, 1970; Bouanchand *et al.*, 1969; Hedges and Datta, 1972; Khatoon and Lyer, 1971). The presence of $fi^+$ R plasmid inhibits the fertility of F factor in *E. coli* K12 whereas the presence of $fi^+$ R plasmids does not. The inhibition of fertility (transfer) of F, $F^{'}$ and Hfr chromosomes in cells containing $fi^{'}R$ is attributed at least partly to the reduced frequency of formation of F-type pili.

Some R plasmids when present in female *E. coli* cells, propagate sex specific phage such as MS2, If1 and 1Ke (Bradly *et al*., 1982; Briton and Beer, 1967; Coetzee *et al*., 1985; Khatoon and Iyer, 1972; Khatoon 1976; Meynell and Lawn, 1968). The R plasmids that confer sensitivity to MS2 are called F-type R plasmids whereas those conferring sensivity to If1 and IKe are referred to as I-type R plasmids and N-type R plasmids respectively.

Another property of the R plasmids that is not of particular importance but can be used for the characterization of R plasmids, includes the property of phage restriction. Bacterial strains bearing some plasmids, including R plasmids, restrict the plaque formation of certain phages to which they are normally sensitive. Specific pattern and extent of phage restriction exhibited by R plasmids bearing stains have been used in the classification of R plasmids by some authors (Bannister and Glover, 1968).

The R plasmid is transferred by conjugation from $F^- R^+$ cells to $F^- R^-$ cells, usually independently of the host chromosome. Transfer starts within 1 minute of mixing the parents Bacteria that have newly received R plasmid pass it on at much higher frequency that those have carried it for many generations (Meynell *et al.*, 1968; Watanabe, 1967).

The current investigation was started to study various genetic properties of R plasmids harboured by Gram negative bacteria of poultry origin in order to characterize them.

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## MATERIALS AND METHODS

**Bacterial Strains:**

Genetically marked bacterial strains and bacteriophages used in these studies are indicated in Table 1 and 2 respectively.

**Standard Plasmids:**

Standard plasmids used in these studies were:

(i) F' pro lac: it was used in the fertility inhibition studies and to propagate F specific phage and was maintained in *E. coli* FPL 5014 sent by Micheal Du Bow of Canada.

RM 98: An R plasmid with N specific sex factor isolated by Khatoon (1976) was used to propagate bacteriophage 1Ke. It was maintained in *E. coli* C600 sent by V.N.lyer of Canada.

(ii) Col Ib: It was used a propagate bacteriophage If1 and was maintained in *E. coli* J53-1 sent by L. Le Minor of France.

**Source of Gram Negative Bacteria:**

Gram negative bacteria were collected from poultry research institute, Korangi, Karachi. The bacteria were obtained as pure culture and were twice purified on MacConkey's medium and maintained on Tryptone agar stabs at 4$^{o}$C. Gram staining and various biochemical tests were performed to identify the cultures.

**Media:**

MacConkey's agar (M.A.) was from Difco or Oxoid (Oxoid MacConkey's agar No. 3). Triple sugar iron agar (T.S.I.) was from Difco Laboratories, USA. Tryptone agar, used for maintaining stock cultures, consisted of Bacto-tryptone 17 grams. Bacto-agar 6 grams; distilled water 1000 ml. L. B. broth, used for growing cultures for conjugation, consisted of: Bacto-tryptone 10 grams. Yeast extract 1 gram; NaCl 8 grams and distilled water 1000 ml. Minimal medium, used in conjugation experiments, consisted of: $NH_4Cl$ 1 gram; $MgSO_4$ 0.13 grams; $KH_2PO_4$ 3 grams; $Na_2HPO_4$ 6 grams; Glucose 4 grams; Agar 20 grams and distilled water 1000 ml.

**Antibiotics:**

The antibiotics used were ampicillin trihydrate (A), chloramphenicol levo (C) gentamycin sulphate (G), kanamycin sulphate (K), neomycin sulphate (N), polymyxin B sulphate (P), streptomycin sulphate (S), and tetracycline hydrochloride (T). All the antibiotics were from Sigma Chemical Co. Ltd. USA.

**Amino Acids/Reagents:**

Amino aicds and thiamine hydrochloride were from Sigma Chemical Co. Ltd. USA. $Na_2HPO_4$, $KH_2PO_4$, NaCl, Glucose and $MgSO_4$ were from E. Merck, Germany.

**Stock Solutions:**

Antibiotic stock solutions (10 mg/ml) were made in distilled water. Solutions of amino acids (4 mg/ml) and thiamine hydrochloride (1 mg/ml) were also prepared in distilled water. The solutions were sterilized by millipore filtration and kept frozen if not in use.

**Bacterial Conjugation:**

The overnight grown cultures of donor and recipients were diluted fifty fold in fresh L.B. broth and grown with shaking at 37$^{o}$C for approximately 1 h so as to given slight turbidity. Donor and recipient were then mixed in a ratio of 1:10 by volume and incubated for two hours at 37$^{o}$C. Controls of unmated donors and recipients were also prepared. After proper incubation, the conjugation mixture as well as controls were centrifuged at 3500 rpm for 30 minutes and the sediment was streaked on M.A. plates containing an appropriate antibiotic (to contraselect recipient) and streptomycin at the level of 500 µg/ml of the medium (to contraselect donor). In most cases, where wild type donors were used the donor was eliminated by using a streptomycin resistant recipient and selecting on high levels of streptomycin. Besides that lactose fermentation character was employed as a mean to identify donors and transconjugants in such a way that with a $lac^{+}$ $str^{s}$ donor $lac^{-}$ $str^{r}$ recipient was used in conjugal cross. The isolated transconjugant colonies were purified twice on MacConkey's medium supplemented with appropriate antibiotics so as to promote the growth of transconjugants only and not the donor and recipient.

In case of conjugal crosses involving R plasmid transfer between auxotrophic standard strains, the conjugation was performed in a similar way. However, after proper incubation (2 h at 37$^{o}$C) the conjugation mixture as well as

controls of donor and recipients were washed twice with saline after spinning them at 3500 rpm and resuspending them in saline followed by centrifugation at 3500 rpm. This process was repeated twice and residue was then streaked on a minimal medium plate containing appropriate nutritional requirement (to contraselect donor) and an appropriate antibiotic (to contraselect recipient).

For quantitative conjugal crosses, the frequency of transfer of a genetic marker was calculated per hundred donors (see below), unless otherwise specified.

$$\text{Frequency of transfer/100 donors} = \frac{\text{Number of transconjugants/ml of conjugation mixture}}{\text{Number of viable donor cells/ml of conjugation mixture}} \times 100$$

**Fertility Inhibition:**

The R plasmids were transferred to *E. coli* 40 bearing F' factor (NJ2-1). Fertility inhibition of *E. coli* NJ2-1 caused by R plasmids was detected by scoring for the donation frequency of F' factor from *E. coli* NJ2-1 to *E. coli* AB712 (F-thr-leu-pro-thi-) in the following conjugal crosses;

*E. coli* NJ2-1X *E. coli* AB 712 (a)

*E. coli* NJ2-1+R X *E. coli* AB712 (b)

Reduction of the transfer frequency of F' factor in cross (b) above, as compared to cross (a), was taken as an indication of fertility inhibition by the R plasmids.

**Phage Propagation:**

Log phase cultures of the bacterial strains were prepared in L-broth. The culture (0.1 ml) was mixed with molten 2 ml L-soft agar which was over laid on the L-agar plates. After the top layer had solidified, one drop of the phage preparation known to give confluent lysis (approximately 1 X $10^5$ pfu/ml) was deposited on the surface. Several drops could be deposited separately on the same plate if the bacterial strain was to be tested for several phages. These drops were dried before incubating the plates at $37^oC$. Lysis was recorded after overnight incubation.

**Bacteriophage Restriction:**

Phage restriction by R plasmid bearing bacteria is a general phenomenon, independent of the fi property of R plasmids (Bannister and Gloves, 1968). R plasmids can, therefore, be classified on the basis of extent and pattern of restriction of a number of test bacteriophages.

Strains of *E. coli* or *S. typhimurium* containing single R plasmids or other plasmids and isogenic controls lacking the plasmid were grown to log phase in L-broth. A bacteriophage to be tested for restriction was plated (by the agar layer method) on isogenic $R^+$ and $R^-$ strains. Restriction was measured as a decrease in efficiency of plating (e.o.p) of a test bacteriophage on $R^+$ host as compared to the e.o.p. of the same phage preparation on $R^-$ control. P22 was plated on *S. typhimurium* HER 1023 $\pm$ R plasmid where as the rest of the phages were plated on *E. coli* $\pm$ R plasmid.

**RESULTS**

R plasmids can be characterized according to the properties they confer on their bacterial hosts. Characterization and classification is necessary if the R plasmids have to be traced epidemiologically. Although there exist several criteria for plasmid classification. R plasmids isolated during these studies were characterized according to the following properties.

a) fertility inhibition by R plasmids
b) propagation of sex specific phages
c) phage restriction
d) frequency of transmission

Total six R plasmids (pFK-2, pFK-6, pFK-7, pFK-8a, pFK-13 and pFK-20) were studied for the properties mentioned above in order to characterize them. Four R plasmids designated as pFK-6, pFK-8a, pFK-13, and pFK-20 showed fertility inhibition and were characterized as $fi^+$, however two R plasmids designated as pFK-2 and pFK-7 did not show fertility inhibition and were, therefore classified as $fi^-$ R plasmids (Table 3). All the six R plasmds i.e. pFK-2, pFK-6, pFK-7, pFK-8a, pFK-13, and pFK-20 when tested by spot lysis did not confer sensitivity to MS2,

M13, f2, If1 and IKe not restricted the phages PIKc, λ, Ǿx174. Mvcts and T6 of *E. coli* and P22 of *S. typhimurium*. Frequency of transmission between *E. coli* strains of these R plasmids was also calculated and was found to be quite high during HFT state (Table 3).

Table 1. Bacterial Strains

| **Strains** | **Genetic Marker or Character** | **Source** |
|---|---|---|
| *E.coli* AB712 | $F^-$ $thr^-$ $leu^-$ $pro^-$ $lac^-$ $thi^-$ $sm^r$ $T6^s$ | E. Adelberg (U.S.A.) |
| *E.coli* FPL 5014 | F` pro lac/Δ pro lac thi $Sm^s$ | M.Du Bow (Canada) |
| *E.coli* Hfr 1 | Host for F specific phages | L.Le Minor (France) |
| *E.coli* NJ 2-1 | F` pro lac/Δ pro lac $trp^-$ $Sm^r$ | N. Jehan (Pakistan) |
| *E.coli* HER 1024 | Propagating strain for T6 | H.W. Ackermann (U.S.A) |
| *E.coli* C600/psu 1 | Prototroph | V.N. lyer (Canada) |
| *S. typhimurium* HER 1023 | Propagating strain for P22 | H.W. Ackermann (U.S.A) |
| *E, coli* J 53-1 | $pro^-$ $met^-$ $nal^r$ | L. Le Minor (France) |

thr = therionine; trp = tryptophane; leu = leucine; r = resistant; lac = lactose
s = sensitive; Sm = streptomycin; Δ = deletion; pro = praline; nal = nalidixic acid

Table 2. Bacteriophage Strains

| **Strains** | **Characteristics** | **Source** |
|---|---|---|
| Ikc | Phage specific for N type plasmids | V.N.lyer (Canada) |
| Ifl | I specific coliphage | L.Le Minor (France) |
| MS2 | F specific coliphage | L.Le Minor (France) |
| M13 | F specific coliphage | K.E. Sanderson (Canada) |
| f 2 | F specific coliphage | K.E. Sanderson (Canada) |
| P22 | Salmonella transducting phage | H.W. Ackermann (Canada) |
| PlKc | Escherichia transducting phage | G. Drapeay (Canada) |
| T6 | Coliphage | H.W. Ackermann (Canada) |
| ǾX174 | Coliphage | H.W. Ackermann (Canada) |
| Λ | Coliphage | H.W. Ackermann (Canada) |
| Mucts | Temperature sensitive mutant of Mu | T. Razzaki (Pakistan) |

Table 3. fi Character and Frequency of R-Plasmids transfer between *E. coli* strains* in LFT and HFT state.

| **R-Plasmids** | **Fi Character**** | **LFT** | **HFT** |
|---|---|---|---|
| pFK-2 | - | $1 \times 10^{+}$ | $1 \times 10^{2}$ |
| pFK-6 | + | $1 \times 10^{0}$ | $1 \times 10^{2}$ |
| pFK-7 | - | $4 \times 10^{-1}$ | $3 \times 10^{1}$ |
| pFK-8a | + | $1 \times 10^{-2}$ | $5 \times 10^{0}$ |
| pFK-13 | + | $0.5 \times 10^{-3}$ | $2 \times 10^{1}$ |
| pFK-20 | + | $1 \times 10^{-2}$ | $2 \times 10^{0}$ |

* The donor in all the crosses was *E. coli* 40 whereas recipient was *E. coli* AB712.
** + = $fi^+$ - = $fi^-$

## DISCUSSION

R plasmids can be divided into $fi^+$ and $fi^-$ groups according to their effect on the F factor associated fertility of *E. coli* $fi^+$ R plasmids can inhibit the F fertility while $fi^-$ does not. Regarding their genetic properties, four out of the six R plasmids studied were found to be $fi^+$ and two $fi^-$. Some R plasmids when present in female *E. coli* cells propagate sex specific phages such as MS2, Ifl, and IKe. The R plasmids that confer sensitivity to MS2 are called F type R plasmids whereas those conferring sensitivity to Ifl and IKe are referred to as I type R plasmids and N type R plasmids respectively. The six R plasmids studied were not found to belong to F, I or N group of plasmids, as they did not confer sensitivity to MS2, Ifl or IKe. It seems that these R plasmids belong to a new group, however,

plasmid specific phages have to be isolated for confirmation. Restriction and modification of phages was first described by Watanabe *et al.* (1964) as the properties peculiar to $fi^+$ R plasmids. Phage restriction was then found to be a property widely distributed among R plasmids irrespective of their fi character. None of six R plasmids studied restricted any phages tested (that is, P22, T6, Plkc, Mucts, λ and ØX174) and probably belong to the group of non phage restricting R plasmids. Frequency of transmission between *E. coli* strains of these R plasmids was also calculated and was found to be quite high during HFT state. This situation is quite alarming from view point of chemotherapy.

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