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R PLASMIDS OF CLINICAL GRAM-NEGATIVE BACTERIA: MOBILIZATION OF NON-CONJUGATIVE R PLASMIDS BY CONJUGATIVE PLASMIDS

Asma Saeed[1], Hajra Khatoon[1]* and Fasihuddin Ahmed Ansari [2]

[1]*Department of Microbiology, University of Karachi, Karachi-75270, Pakistan*
[2]*Division of Reproduction and Endocrinology, School of Biomedical and Health Sciences, King's College, London, England*

ABSTRACT

During studies on the transferable antibiotic resistance of clinical gram-negative bacteria, 28 R plasmids were isolated from indigenous strains. The R plasmids conferred resistance to one or several antibiotics on their host strains and most of them were conjugally transferable to other pathogenic bacteria, posing a threat to chemotherapy. Some of the R plasmids were conjugally non-transferable. It was of interest to see whether they could become transferable to other bacteria in the presence of a co-resident conjugative plasmid. For this purpose, genetically marked strains of *Escherichia coli*, carrying the individual, non-transferable R plasmids, were conjugally infected by the conjugative plasmids: F′ *pro*$^+$ *lac*$^+$, KR61-KNST, pSK1a, pAK3, pAK6, pAK13 and pAK17. Mobilization of the non-transferable R plasmids was observed in different patterns by the co-existing conjugative plasmids in conjugal crosses.

Key-words: R plasmids, mobilization, conjugative plasmids, non-conjugative plasmids, gram-negative bacteria, antibiotic resistance.

INTRODUCTION

R Plasmids or R factors are extra chromosomal DNA structures that confer resistance to one or several antibiotics/drugs on their bacterial hosts thus creating problems in chemotherapy (Davies, 1981). The R plasmid mediated antibiotic/drug resistance spreads in bacterial populations by cell contact or conjugation (Meynell *et al.*,1968; Novick, 1969). Non-pathogenic bacteria bearing R plasmids are equally dangerous because they can transfer their R plasmids, along with all their resistances, to the pathogenic bacteria. Infectious drug resistance, therefore, constitutes a serious threat to public health. Since its discovery in Japan in 1959 (Akiba, 1959; Ochiai *et al*., 1959), this type of drug resistance has been detected in many other countries (Degner *et al*., 1983; Farrar, 1981; Fenoll *et al*.,1987; Gosling, 1986; Khatoon, 1971; Olarte, 1981).

R plasmids can be divided into two groups: conjugally transferable or conjugative and conjugally non-transferable or non conjugative (Kilbane and Malamy, 1980; Willets and Crowther, 1981). The non-conjugative plasmids can be occasionally mobilized in the presence of a conjugative R plasmid (Fekete and Frost, 2000; Willets and Crowther, 1981). Mobilization of non-conjugative plasmids is of great importance not only in bacterial genetics but also in clinical medicine. In this way the resistances that were originally non-transferable become conjugally transferable to pathogenic bacteria and can create further problems in chemotherapy.

A conjugative R plasmid, KR61-KNST, which conferred resistance to kanamycin, neomycin, streptomycin and tetracycline (KNST), and was conjugally transferable to (& from) *Escherichia*, *Salmonella*, *Aerobacter* and *Shigella* has been reported by Khatoon (1971) and Khatoon and Ali Muhammad (1986). KR61-KNST could mobilize a non-conjugative R plasmid, KR61 A, carrying resistance to ampicillin and could cause its conjugal transmission to all the above bacterial hosts (Khatoon,1971; Khatoon and Ali Muhammad,1986). Similarly, at least two conjugative plasmids pSK1-a & pAK17 carrying KNT resistances have been isolated in our lab (Saeed, 2003) that could mobilize KR61-A.

The current investigation was started to study the nature of R plasmids present in local bacterial population of clinical gram-negative bacteria. A total of 229 bacteria were studied for the presence of conjugative and non-conjugative R plasmids. It was of interest to see whether KR61-KNST which mobilized KR61-A (Khatoon,1971; Khatoon and Ali Muhammad,1986) could also mobilize the non-conjugative plasmids existing in the local bacterial populations. Similarly, some conjugative plasmids isolated during these studies and F′pro$^+$lac$^+$, which also mobilized KR61 A (Saeed, 2003), were studied for their ability to mobilize non-conjugative plasmids existing in the local bacterial populations. Mobilization of other non-conjugative R plasmids, isolated else where in the world e.g. pBR322, pBR325, pUT1334, pEKA28, pBK1, by the above mentioned conjugative plasmids was also studied.

Corresponding author: Dr. Hajra Khatoon; Department of Microbiology, University of Karachi, Karachi-75270, Pakistan. Phone: 0092-300-2455775. Fax: 0092-21-5688842. E-mail: hajrakhatoon@hotmail.com

MATERIALS AND METHODS

Bacterial strains

The standard bacterial strains used in the study are listed in Table 1.

Table 1. Bacterial strains.

Strains*	Genotype**	Source
E.coli 13-6a	F⁻ *lac*$^+$ *proA ade trp met str*^{r} *tsx*^{r}	R. Iyer
E.coli 40 MD	F⁻ Δ(*pro-lac) trp str*^{r}	M. DuBow
E.coli FPL5014	F′ *pro*$^+$*lac*$^+$ / Δ(*pro-lac) thi str*^{s}	A. I. Bukhari
E.coli AB712	F⁻ *thr leu thi pro lac str*^{r}	Mary Berlyn
S.typhimurium, LT2, 153K	prototroph, carrying the R plasmid KR61-KNST	H. Khatoon

**E.coli= Escherichia coli*, *S. typhimurium= Salmonella typhimurium*. KR61-KNST carried resistane to kanamycin(50μg/ml of the medium), neomycin(100μg/ml), streptomycin(100μg/ml) and tetracycline(50μg/ml).
***str*^{r} & *str*^{s}=resistance & sensitivity to streptomycin respectively, *tsx*^{r}=resistance to the bacteriophage T6.

Table 2. Standard non-conjugative bacterial plasmids used in the study.

Plasmid	Resistances*	Strain**	Source
pBR322	AT	GC875 DH5□	G. Chaconas (Canada)
pBR325	ACT	GC876 DH5□	G. Chaconas (Canada)
pUT1334	ASu	RM4028	R. J. Meyer (USA)
pEKA28	AT	EKA753	D. H. Figurski (USA)
pBK1	AC	EKA844	D. H. Figurski (USA)
KR61-A	A	13-6a	H. Khatoon (Canada)

*A=ampicillin, C=chloramphenicol, Su=sulfonamide and T=tetracycline.
**All the strains were *Escherichia coli* K12 strains.

Standrad bacterial plasmids

The standard non-conjugative bacterial plasmids used in the study are listed in Table 2 .
The standard conjugative plasmids used in the study included F′pro$^+$lac$^+$ (carried by *E.coli* FPL5014) and KR61-KNST (that was carried by *E.coli* 13-6a host or *Salmonella typhimurium* LT2 153K host) that had resistance to kanamycin (50μg/ml), neomycin (100μg/ml), streptomycin (100μg/ml) and tetracycline (50μg/ml).

Sources of gram-negative bacteria

Gram-negative bacteria were obtained from hospitals or pathological labs of Karachi.

Antibiotics

The antibiotics used were: ampicillin trihydrate (A), chloramphenicol (C), gentamycin sulfate (G) , kanamycin sulfate (K) , neomycin sulfate (N), streptomycin sulfate (S) and tetracycline hydrochloride (T). All the antibiotics were from Sigma Chemical Company, U.S.A.

Table 3. Mobilization patterns of non-conjugative plasmids with the co-resident KR61-KNST plasmid.

Non-conjugative plasmid* & its original resistance(s)	Resistances transferred**	Mobilization pattern
KR-61A (A)	A	Mobilized alone
	AKNST	Mobilized with KR61-KNST
pMT14 (AST)	AKNST	Mobilized with KR61-KNST
pZ26 (A)	A	Mobilized alone
	AKNST	Mobilized with KR61-KNST
pBK1 (AC)	ACKNST	Mobilized with KR61-KNST
pBR322 (AT)	AKNST	Mobilized with KR61-KNST
pBR325 (ACT)	ACKNST	Mobilized with KR61-KNST
pEKA28 (AT)	AT	Mobilized alone
	AKNST	Mobilized with KR61-KNST
pUT1334 (ASu)	ASu	Mobilized alone

*All the plasmids were in an *Escherichia coli* host that carried a co-resident KR61-KNST plasmid.
**A=ampicillin, C=chloramphenicol, K=kanamycin, N=neomycin, S=streptomycin, Su=sulfonamide and T=tetracycline.

Table 4. Mobilization patterns of non-conjugative plasmids with the co-resident pSK1a (KNT) plasmid.

Non-conjugative plasmid* & its original resistance(s)	Resistances transferred**	Mobilization pattern
KR61-A (A)	A	Mobilized alone
pMT14 (AST)	AKNST	Mobilized with pSK1a
pZ26 (A)	A	Mobilized alone
pBR322 (AT) & pBR325 (ACT)	_	Not mobilized
pUT1334 (ASu)	AKNSuT	Mobilized with pSK1a
pBK1 (AC)	ACKNT	Mobilized with pSK1a

*All the plasmids were in an *Escherichia coli* host that carried a co-resident pSK1a (KNT) plasmid.
**A=ampicillin, C=chloramphenicol, K=kanamycin, N=neomycin, S=streptomycin, Su=sulfonamide and T=tetracycline.

Table 5. Mobilization patterns of non-conjugative plasmids with the co-resident F′ *pro*$^+$ *lac*$^+$ plasmid.

Non-conjugative plasmid* & its original resistance(s)	Resistances transferred**	Mobilization pattern
KR61-A (A)	A	Mobilized alone
	A *pro*$^+$ *lac*$^+$	Mobilized with F′ *pro*$^+$ *lac*$^+$
pZ26 (A)	A	Mobilized alone
	A *pro*$^+$ *lac*$^+$	Mobilized with F′ *pro*$^+$ *lac*$^+$
pBK1 (AC) & pEKA28 (AT)	_	Not mobilized
pBR322 (AT)	AT *pro*$^+$ *lac*$^+$	Mobilized with F′ *pro*$^+$ *lac*$^+$
pBR325 (ACT)	ACT *pro*$^+$ *lac*$^+$	Mobilized with F′ *pro*$^+$ *lac*$^+$
pUT1334 (ASu)	ASu	Mobilized alone
	ASu *pro*$^+$ *lac*$^+$	Mobilized with F′ *pro*$^+$ *lac*$^+$

*All the plasmids were in an *Escherichia coli* host that carried a co-resident F′ *pro*$^+$ *lac*$^+$ plasmid.
**A=ampicillin, C=chloramphenicol, Su=sulfonamide and T=tetracycline.

Table 6. Mobilization patterns of non-conjugative plasmids with the co-resident pAK17 (KNT) plasmid.

Non-conjugative plasmid* & its original resistance	Resistances transferred**	Mobilization pattern
KR61-A (A)	A	Mobilized alone
	AKNT	Mobilized with pAK17
pZ26 (A)	AKNT	Mobilized with pAK17

*All the plasmids were in an *Escherichi coli* host that carried a co-resident pAK17 (KNT) plasmid.
**A=ampicillin, K=kanamycin, N=neomycin and T=tetracycline

Media

Resistance determinations were made on MacConkey's Agar (Difco), to which single antibiotics were added at desired concentrations (usually 100μg/ml) as has been described earlier (Amir Ali and Khatoon, 1976; Khatoon, 1976). Minimal Inhibitory concentrations (MICs) of the standard bacterial strains, used as recipients in conjugal crosses, were determined as described by Amir Ali and Khatoon (1976) and Jahan (1991). All the standard strains were inhibited at the concentration of 30μg of antibiotic per ml of the medium, for all antibiotics. The *E. coli* strains 13-6a, 40 MD and AB712 had high level, chromosomal streptomycin resistance and could resist more than 500μg/ml.

For conjugation experiments, bacterial cultures were grown in L.B. broth (Khatoon, 1976; Khatoon and Ali Muhammad,1986) or Antibiotic Medium No.3, Oxoid. Conjugal crosses were carried out by the broth method as described earlier (Khatoon, 1976). The transconjugants were selected on Minimal Agar or on MacConkey's Agar,

depending on the nature of the conjugal cross. Minimal Agar had the same composition as that of Davis Minimal Agar (Difco).

Construction of strains with double plasmids

The strains carrying two plasmids i.e. the mobilizing plasmid and the plasmid to be mobilized were constructed by the following methods.

In case of R plasmids or strains bearing resistance determinants the doubly infected strains were constructed by selecting on a medium containing an antibiotic (e.g. ampicillin) of which the resistance was carried by one plasmid and another antibiotic (e.g. kanamycin or neomycin or tetracycline) of which the resistance was carried by the other plasmid. By this method the mobilizing plasmid was transferred conjugally to a strain bearing potentially mobilizable plasmid.

In case of the strains bearing an F′*pro*$^+$*lac*$^+$ (as a mobilizing plasmid) and a resistance / or plasmid to be mobilized, *E.coli* FPL5014 bearing F′*pro*$^+$*lac*$^+$ was conjugated with the strain bearing mobilizable plasmid and F′*pro*$^+$*lac*$^+$ was conjugally transferred to this strain by looking for *lac*$^+$ colonies (on MacConkeys medium carrying an antibiotic) of a *lac*$^-$ antibiotic resistant recipient cell.

In case of a strain bearing F′*pro*$^+$*lac*$^+$ and KR61-A, the strain construction was carried out as follows : *E.coli* 13-6a bearing KR61-A + KR61-KNST was conjugated to *E.coli* FPL5014 bearing F′*pro*$^+$*lac*$^+$. Selection was made on the minimal medium containing thiamine & ampicillin. In this way KR61-A was transferred (by being mobilized with KR61 KNST) to *E.coli* FPL5014. Transconjugants were selected that carried F′*pro*$^+$*lac*$^+$ and KR61-A alone without KR61-KNST. This was tested by replication of the transconjugants on MacConkey's plates containing individual antibiotics: ampicillin, kanamycin, neomycin, streptomycin and tetracycline.

RESULTS

Screening for antibiotic resistance and isolation of R plasmids

A number of 229 gram negative bacteria, collected from clinical sources were screened for their resistance to ampicillin(A), chloramphenicol (C), gentamycin(G), kanamycin(K), neomycin(N), streptomycin(S) and tetracycline (T). Of the 229 bacteria, 158 were found resistant to one or more antibiotics. The resistances were tested at a level of 100 μg / ml of the medium. The bacteria included species of *Salmonella, Shigella, Klebsiella, Aeromonas, Enterobacter, Escherichia, Proteus* & some other unidentified organisms.

The resistant bacteria (potential R plasmid donors) were conjugated to standard *E.coli.* K-12 recipients: 13-6a, 40MD or AB712 for the conjugal transfer of their resistances. Resistance to streptomycin could only be ascertained after transfer of the R plasmid to *E.coli* FPL5014, that was streptomycin sensitive. Total 28 R Plasmids were isolated including pSK1a, pAK3, pAK6, pAK13 & pAK17, that were used in the mobilization studies. pSK1a & pAK17 carried resistances KNT, where as pAK3 & pAK13 had ampicillin (A) resistance & pAK6 had ampicillin & streptomycin (AS) resistance. All the resistances were present at the level of 100μg / ml of the medium.

Non-conjugative plasmids

The non-conjugative plasmids that could be mobilized in conjugations by conjugative R plasmids included pMT14 (detected in a clinical strain of *E.coli*) carrying resistances to ampicillin, streptomycin & tetracycline (AST) & pZ26 (detected in a clinical strain of *Salmonella typhi*) carrying resistance to ampicillin (A). All the resistances were present at the level of 100μg / ml of the medium.

Mobilization of non-conjugative plasmids by conjugative plasmids

Of the conjugative plasmids, KR61-KNST, pSK1a, pAK17, pAK3, pAK6, pAK13 & F′*pro*$^+$*lac*$^+$ were used for mobilizing the co-existnig non-conjugative plasmid. The mobilization patterns of various non-conjugative plasmids by KR61-KNST, pSK1a, F′*pro*$^+$*lac*$^+$ and pAK17 can be seen in Tables 3, 4, 5 & 6 respectively.

The conjugative plasmids pAK3, pAK6 and pAK13 were not found to mobilize any non-conjugative plasmid.

DISCUSSION

Of the 229 gram negative bacteria collected from clinical sources, 70 were tested for the transferability of their resistances and 22 were found to transfer their resistances by conjugation (or contained conjugative R plasmids). This indicates that at least 31% of clinical gram negative bacteria in Karachi possess

conjugative R plasmids. The percentage may be even higher since some of the conjugations did not work because of technical difficulties. Considering their ability of conjugal transfer, conjugative R plasmids pose a great threat to chemotherapy even if they are carried by non-pathogenic bacteria.

Mobilization of non-conjugative plasmids by conjugative plasmids can further create problems in chemotherapy. It was, therefore, of interest to study the mobilization ability of the conjugative plasmids. Therefore, mobilization ability of KR61-KNST, pSK1a, F′*pro*$^+$*lac*$^+$, pAK17, pAK3, pAK6 & pAK13 was studied. The non-conjugative plasmids studied for mobilization included the indigenous plasmids pMT14 & pZ26 & other plasmids pBR322, pBR325, pUT1334, pEKA28, pBK1 & KR61-A.

According to the results presented here, KR61-KNST appears to be the most efficient plasmid regarding its mobilization ability since it mobilized all the above mentioned non-conjugative plasmids either alone or in combination with itself (Table 3). The mechanism of transfer has yet to be elucidated. However, it was of interest to see that pBR322 & pBR325 were transferred in combination with KR61-KNST & not alone. This seems to indicate that KR61-KNST probably mobilizes these plasmids in a manner similar to F factor as described by Kilbane and Malamy (1980) i.e. via transposon-mediated recA-independent fusion. Other plasmids that were transferred only in association with KR61-KNST included pMT14 & pBK1. The non-conjugative plasmids mobilized alone included KR61-A, pEKA28, pZ26 & pUT1334. It may be that these non-conjugative plasmids possess *oriT* and some other genes but lack transacting functions necessary to activate these genes as suggested by Leemans *et al.* (1981). These functions are probably provided by KR61-KNST.

Another conjugative plasmid, pSK1a, isolated from a clinical, indigenous srain of *Salmonella typhi* para B, was not able to mobilize pBR322 and pBR325 indicating that it had some structural dissimilarity from KR61-KNST (Table 4). The non-conjugative plasmids mobilized alone by pSK1a included KR61-A, pEKA28, pZ26 i.e., the plasmids also mobilized alone by KR61-KNST. Thus pSK1a shared this similarity with KR61-KNST. However the plasmid pUT1334 that was mobilized alone by KR61-KNST was mobilized by pSK1a in combination with itself and not alone. Other plasmids mobilized in this manner included pMT14 and pBK1.

F′*pro*$^+$*lac*$^+$ mobilized the plasmids pBR322 and pBR325 in combination with itself and not alone (Table 5). These findings seem to support the findings of Kilbane and Malamy (1980), who reported that pBR322 was mobilized via transposon-mediated recA- independent fusion. The same mechanism may also operate for pBR325. the non-conjugative plasmids mobilized alone included KR61-A, pZ26 and pUT1334 i.e. the same plasmids also mobilized alone by KR61-KNST. However the plasmid pEKA28 mobilized alone by KR61-KNST was not mobilized by F′*pro*$^+$*lac*$^+$ and similarly pBK1 was also not mobilized.

Another conjugative R plasmid, pAK17, isolated from a clinical indigineous strain of *E.coli* was not able to mobilize pBR322, pBR325, pUT1334 and pEKA28 indicating its gross dissimilarity with the plasmids KR61-KNST, pSK1a and F′*pro*$^+$*lac*$^+$ (Table 6). However pAK17 mobilized pZ26 in combination with itself and also mobilized KR61-A either alone or in combination with itself.

Other conjugative R plasmids isolated from indigenous clinical bacteria, designated as pAK3, pAK6, and pAK13 (all of which carried ampicillin resistance) were not able to mobilize any of the non-conjugative plasmids mentioned above nor the resistances of *E.coli* AS-60 (which carried resistance to CGKNT) and *E.coli* AS-87 (which carried resistance to GKN) indicating that they probably lacked the mobilization ability.

It is interesting to note that the R plasmids which caused mobilization of non conjugative plasmids carried either KNST (kanamycin, neomycin, streptomycin and tetracycline) resistances or KNT resistances. KR61-KNST carried KNST resistances whereas pSK1a and pAK17 both carried KNT resistances. Whether this has any connection with mobilization ability would be interesting to investigate.

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