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TRANSFER AND BEHAVIOUR OF SOME R PLASMIDS IN PLANT ASSOCIATED BACTERIA

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Abstract

Eleven R plasmids isolated from enteric bacteria were studied for their transfer and stability in plant associated bacteria to use them as vehicles for *In vivo* genetic engineering. The bacteria of plant origin viz., *Xanthomonas*, *Erwinia*, *Citrobacter*, *Rhizobium*, *Agrobacterium* and *Pseudomonas* used as recipients and the R plasmids used included pFK2, pFK6, pFK7, pFK8, pFK20, pAS72, pNJ15, pNJ18, pFK5a, pFK17 and pFK18. The R plasmids which carried resistance to one or five different antibiotics were tested for their conjugal transmission from enteric bacteria to the bacteria of plant origin. Two of these R plasmids viz., pFK6 and pFK7 could be conjugally transmitted to four different bacteria of plant origin whereas, pFK2 could be transmitted to three and the rest could be transmitted to two or one plant associated bacteria. Most of the R plasmids were found to be stably maintained after their transmission into the bacteria of plant origin indicating that they could be used as vehicles for *In vivo* genetic engineering of these bacteria.

Introduction

In vivo genetic engineering deals with the manipulation of the genome of a cell while it is still alive by purely genetical means and thus one can introduce new characters into a cell or can change or remove the existing characters (Howe *et al.*, 1975; Bukhari *et al.*, 1977; Denarie *et al.*, 1977). The necessary tools for *In vivo* genetic engineering include extrachromosomal elements or plasmids and transposons (Bukhari *et al.*, 1977; Bukhari, 1979; Murooka, 1981).

The cells of gram negative bacteria normally carry a single circular chromosome that carries all the essential genes required for survival (Bachmann, 1983; Sanderson *et al.*, 1983; Leary *et al.*, 1982). Certain bacteria, however, contain other self replicating DNA structures referred to as extrachromosomal elements, plasmids or episomes (Novick, 1969; Old *et al.*, 1980; Jahan *et al.*, 1997). The plasmids can be of various types, including R plasmid that carry genes for antibiotic resistance and are thus very useful in genetic studies (Old *et al.*, 1980). There exist two types of R plasmids (i) conjugative or complete and (ii) nonconjugative or incomplete (Novick, 1969). The conjugative R plasmids can be transmitted, alongwith all their resistant genes, from one bacterial cell to another by conjugation (Novick, 1969; Jahan *et al.*, 1997). Usually the host range of plasmids is rather limited where they are conjugally transmitted to the species of the same genus or to the species of closely related genera. However, there are few exceptional cases where plasmids can be transferred to a wide variety of bacterial genera and such R plasmids are referred to as broad-host-range plasmids (Denarie *et al.*, 1977; Old *et al.*, 1980).

Occasionally, a gene from a plasmid can jump from its place and be inserted into certain other DNA structure such as another plasmid or a phage that might be present into the cell or into the chromosome thus causing a genetic alteration. Such a gene referred to as a jumping gene or a transposon can be made use of in manipulating the genome of a bacterial cell (Bukhari *et al.*, 1977; Bukhari, 1979). The genetic alterations or rearrangements caused by transposons include mutations, deletions, inversions, fusions and recombinations (Howe *et al.*, 1975; Bukhari, 1979). These changes can be brought about in a desired bacterial cell only if the transposon can be introduced in the cell. The only method of introducing a transposon into a cell is to insert the transposon into a broad-host-range plasmid in a genetically marked host bacterium such as *Escherichia coli* followed by conjugal transfer of the plasmid + transposon complex into the desired bacterial cell. If one wishes to perform *In vivo* genetic engineering in plant associated bacteria, one must have broad-host-range plasmids that are conjugally transferable to these bacteria and transposons that can express their functions in such bacteria. Studies were therefore carried out to see whether the R plasmids can be used as vehicles to transport transposons into plant associated bacteria. The results on eleven different R plasmids have been reported here.

Materials and Methods

Media: For broth cultivation of bacteria, L.B. broth containing Bactotryptone 10 g, yeast extract 5 g, NaCl 10 g, glucose 10 g, in 1000 ml distilled water was used. The pH was adjusted to 7 before autoclaving. L.B. agar was made by adding 1.5% agar to L.B. broth. All plant associated bacteria could be grown on L.B. agar. Rhizobia could also be grown on yeast extract mannitol agar as described by Chatterjee (1980). The enteric bacteria were grown on MacConkey's agar. All the purified cultures were picked and stored in tryptone agar stabs containing Bactotryptone 17 g, Bactoagar 5 g in 1000 ml distilled water at 4°C.

Standard cultures: The standard cultures used as recipients of R plasmids included the following:

1. *Xanthomonas malvacearum*: from Dr. M.A.R. Bhatti, Agriculture University, Faisalabad.
2. *Xanthomonas campestris*: from Dr. Muhammad Aslam of N.A.R.C., Islamabad.
3. *Erwinia chrysanthemi* AC4075: from Dr. Arun K. Chatterjee, Kansas State University, U.S.A.
4. *Agrobacterium tumefaciens*: from Dr. R.A. Lelliott, National Collection of Plant Pathogenic Bacteria, Ministry of Agriculture, Fisheries and Food, U.K.
5. *Rhizobium*: from Dr. Richard Griffin, Nitrogen Fixation and Soybean Genetic Research Laboratory, United States Department of Agriculture, U.S.A.
6. *Pseudomonas syringae*: from Dr. R.A. Lelliott, National Collection of Plant Pathogenic Bacteria, Ministry of Agriculture, Fisheries and Food, U.K.
7. *Citrobacter freundii* ATCC 8090: from American Type Culture Collection, USDA, Beltsville, U.S.A.

8. *Escherichia coli* BU40: (F' ▲ *prolac*, *trp*⁻, *str*ʳ) from Dr. A.I. Bukhari, Cold Spring Harbor, New York, U.S.A.
9. *Escherichia coli* AB712: (F' *lac*⁻, *leu*⁻, *thr*⁻, *pro*⁻, *str*ʳ) from DR. E. Adelberg, Yale University, U.S.A.

R plasmids: The R plasmids used in the studies were isolated in our lab and are listed in Table 1. Original host bacteria from which the R plasmids were isolated are also listed in Table 1. The R plasmids used in the current studies were present either in *E. coli* BU40 or *E. coli* AB712. All the R plasmids were freely transferable by conjugation between *E. coli* BU40 and *E. coli* AB712.

Table 1. R plasmids, their original host bacteria and resistance patterns.

R plasmid designation	Original host	Resistance pattern of the R plasmid*
pFK2	*Salmonella*	KN
pFK6	*Escherichia*	KST
pFK7	*Escherichia*	KNST
pFK8	*Klebsiella*	AST
pFK20	*Escherichia*	AKNS
pAS72	*Escherichia*	ACGKN
pNJ15	*Escherichia*	ACKNT
pNJ18	*Escherichia*	ACKNT
pFK5a	*Escherichia*	A
pFK17	*Escherichia*	T
pFK18	*Escherichia*	KNT

*A = ampicillin, C = chloramphenicol, G = gentamycin, K = kanamycin, N = neomycin, S = streptomycin, T = tetracycline. The level of antibiotic resistance was 100 μg/ml in each case.

Antibiotics: The antibiotics used were ampicillin trihydrate (A), gentamycin sulfate (G), chloramphenicol (C), kanamycin sulfate (K), neomycin sulfate (N), streptomycin sulfate (S) and tetracycline hydrochloride (T). Antibiotic stock solutions were made in distilled water in the concentration of 10 mg/ml. Chloramphenicol was dissolved in ethanol. All the solutions were sterilized by filtration through bacteriological filters and kept refrigerated at 4°C. Solutions not in use were kept frozen.

Sensitivity levels (MICs) of recipients: Overnight broth cultures of the recipient strains were plated in 0.1 ml amounts on L.B. agar containing known graded concentrations of each antibiotic to determine the minimal inhibitory concentration (MIC) of the latter capable of totally annihilating the recipient cells. Growth (or its absence) was recorded after incubation at 30°C for plant associated bacteria and 37°C for enteric bacteria.

Conjugation experiments: The cultures of donor and recipient cells were grown overnight in L.B. broth. Next morning the overnight culture was diluted 50 fold in L.B. broth and grown with shaking at 30°C or 37°C depending on the type of organism for 30 minutes or till slight turbidity was visible. Donors were then mixed with recipients in the proportion of 1:10 and incubated at 30°C in case of plant associated bacteria or at 37°C in case of enteric bacteria for the desired length of time. If the cross involved a plant pathogenic and an enteric bacterium, the incubation was carried out at 30°C for six hours as described by Chatterjee (1980). In case the donor and recipient both were enteric bacteria, the incubation was carried out at 37°C for two hours as described by Khatoon *et al.*, (1998). Controls which consisted of the donor and recipient cells were treated similarly.

After proper incubation the conjugation mixture was plated on selective medium containing appropriate antibiotic and/or nutritional markers to contraselect recipient and in some cases also donors. Control platings of unmated donors and recipient cells were always made. In case of a cross between two enteric bacteria, transconjugants appeared after an incubation of 24 hours at 37°C, whereas in case of a cross between an enteric and a plant associated bacterium transconjugants appeared in 4 days following incubation at 30°C. Transconjugant colonies arising on the plated media were purified twice on the selective medium used and replicated to check for the donated markers. Sometimes solid method of conjugation (plate conjugation), as described by Chatterjee (1980), was used if the above method of conjugation failed.

Spontaneous segregation: To determine the stability of R plasmids in plant associated bacteria, the spontaneous segregation of these R plasmids was studied. Strain bearing the R plasmid to be studied was grown in antibiotic free L.B. broth through six consecutive transfers with 24 hours intervals and a 20-fold dilution each time. Finally the culture was diluted and plated on L.B. agar to obtain isolated colonies. Some 200 colonies were then gridded on to master plates which were then replicated on the antibiotic containing L.B. agar plates to check for loss/stability of resistance determinants. Antibiotic resistant and sensitive control strains were always replicated along with the colonies of the test strain.

Results

Transfer of R plasmids to plant associated bacteria: The results showed that some R plasmids could be transmitted to as many as four different plant associated bacteria. pFK2 was transferred to *Xanthomonas*, *Rhizobium* and *Agrobacterium*. pFK6 could be transmitted to *Xanthomonas*, *Erwinia*, *Rhizobium* and *Agrobacterium*. pFK7 was transferred to *Xanthomonas*, *Erwinia*, *Citrobacter* and *Rhizobium*. pFK8 was transferred to *Xanthomonas* and *Agrobacterium*. pFK20, pFK5a and pFK17 were transferred to *Xanthomonas* and *Rhizobium*. pAS72 was transferred to *Erwinia* and *Agrobactrium*. However, its transmission to *Agrobacterium* was abortive as the resistances were lost upon purification of the transconjugants. Similarly, transmission of pNJ15 and pNJ18 to *Agrobacterium* was abortive, however, pNJ15 was stably maintained in *Rhizobium*. pFK18 was also transferable and maintained in *Rhizobium* (Table 2).

Table 2. Conjugal transfer of R plasmids to plant associated bacteria.

R plasmid	*Xanthomonas*	*Erwinia*	*Citrobacter*	*Rhizobium*	*Agrobacterium*	*Psuedomonas*
pFK2	+	-	-	+	+	-
pFK6	+	+	-	+	+	-
pFK7	+	+	+	+	-	-
pFK8	+	-	-	-	+	-
pFK20	+	-	-	+	-	-
pAS72	-	+	-	-	(+)	-
pNJ15	-	-	-	+	(+)	-
pNJ18	-	-	-	-	(+)	-
pFK5a	+	-	-	+	-	-
pFK17	+	-	-	+	-	-
pFK18	-	-	-	+	-	-

+ = Presence of conjugal transfer.
- = Absence of conjugal transfer.
(+) = Abortive transfer.

Stability of R plasmids in plant associated bacteria: For performing *In vivo* genetic engineering in plant associated bacteria, it is necessary that the plasmids be stably maintained inside these hosts. A study was therefore made to see whether the plasmids were stably maintained or any loss occurred due to spontaneous segregation as might occur in case of some plasmids (Novick, 1969).

Most of the R plasmids were either completely or partly stable in the plant associated bacteria. pFK2 was completely stable in *Xanthomonas*, *Rhizobium* and *Agrobacterium*. pFK6 was stable in *Xanthomonas* and *Agrobacterium* and partly stable in *Rhizobium*. pFK17 and pFK20 were stable in *Xanthomonas* and *Rhizobium*. pNJ15, pFK5a and pFK18 were stable in *Rhizobium*. pFK8 was stable in *Agrobacterium*. From these results it appears that there exists a possibility of using these R plasmids as vehicles for *In vivo* genetic engineering of the plant associated bacteria in which they are stably maintained (Table 3).

Discussion

Eleven R plasmids, isolated from enteric bacteria in our lab were studied for their conjugal transfer to plant associated bacteria. All the plasmids that were transmitted to plant associated bacteria, expressed all their resistance markers indicating that the new hosts had no adverse effect on their expression/replication. Except *Agrobacterium*, where inheritance of three plasmids was abortive, inheritance was stable in other cases.

Stability of the inherited R plasmids was further checked by spontaneous segregation of the plasmids in plant associated bacteria (Table 3). No complete loss of any R plasmid occurred even by repeated cultivation in antibiotic free broth for six consecutive days. A number of plasmids were completely stable whereas some were partly stable in their new hosts except for *Erwinia* and *Citrobacter* where partial stability was observed.

Table 3. Stability of R plasmids in plant associated bacteria.

Host bacterium	Plasmid	Resistance markers	Percent loss of resistance* on the plasmid
Xanthomonas	pFK2	KN	No loss of any resistance
"	pFK6	KST	No loss of any resistance
"	pFK7	KNST	K(73), N(69), S(16.5), No loss of T
"	pFK8	AST	A(3.5), S(3), T(6)
"	pFK20	AKNS	No loss of any resistance
"	pFK5a	A	A(5.5)
"	pFK17	T	No loss
Erwinia	pAS72	ACGKN	C(36), G(56), No loss of A, K, N
Citrobacter	pFK7	KNST	S(100), No loss of K, N, T
Rhizobium	pFK2	KN	No loss of any resistance
"	pFK6	KST	T(30.5), No loss of K, S
"	pFK7	KNST	T(18.5), No loss of K, N, S
"	pFK20	AKNS	No loss of any resistance
"	pNJ15	ACKNT	No loss of any resistance
"	pFK5a	A	No loss
"	pFK17	T	No loss
"	pFK18	KNT	No loss of any resistance
Agarobacterium	pFK2	KN	No loss of any resistance
"	pFK6	KST	No loss of any resistance
"	pFK8	AST	No loss of any resistance

*The number of colonies tested was 200 in each case.

Percent loss is indicated by numbers in brackets.

Note: Two cultures of *Erwinia* (carrying pFK6 and pFK7) died and could not be studied.

During these studies, it was noted that no complete loss of any plasmid occurred. Two of these R plasmids could be used for as many as four plant associated bacteria, whereas others could be used for one, two or three plant associated bacteria. Some of these R plasmids, at least those that are transferred to three or four genera, could well be regarded as broad-host-range plasmids. Such plasmids can be a very useful tool for *In vivo* genetic engineering and search for some more broad-host-range plasmids is still in progress (Bagadasarian, 1981; Gotz *et al.*, 1996; Blanty *et al.*, 1997; Sobecky, 1998).

Gene manipulation in plants requires a suitable host that can transfer a cloned gene to different plants. The Ti plasmid of *Agrobacterium tumefaciens* can be used for this purpose but large size of the plasmid (200 Kb) and ability of *Agrobacterium* to infect dicotyledonous plants only, are the barriers in its use as a vector in plant cells (Bevan & Chilton, 1982; Chilton; 1983; Klee *et al.*, 1987). Most of the economically important crops are monocotyledonous eg., maize, rice and barley etc., other techniques are

being developed to introduce a desired DNA into these plants for example direct gene transfer, electroporation etc., (Rhodes *et al.*, 1988). Besides *Agrobacterium*, plasmids of certain other plant associated bacteria are also under investigation eg., *Xanthomonas* (Amuthan & Mahadevan, 1994).

It therefore seems important to search for a broad-host-range plasmid that could be transferred to different plant associated bacteria. Such a plasmid becomes more useful if it is containing an antibiotic resistance marker specially tetracycline which is being extensively used these days in plant systems. The Kan^r genes from *E. coli* transposon Tn5 has been used as a selective marker in plants (Shah *et al.*, 1987; Weising *et al.*, 1988; Quentmeier & Friedrich, 1994).

Since most of our R plasmids are carrying a kanamycin resistance marker on them, one can use them as a suitable vector for gene manipulation in various plant systems employing the already existing transposons such as bacteriophage Mu (Howe *et al.*, 1975; Bukhari, 1979; Murooka *et al.*, 1981).

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