Frequent loss of a native giant linear plasmid from UV-induced tetracycline sensitive isolates of producer *Streptomyces rimosus* strain

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A tetracycline producer strain of *Streptomyces rimosus* containing a large linear (255 kb) plasmid was exposed to UV radiation to induce *tet*-resistant (*tet*^{r}) → *tet*-sensitive (*tet*^{s}) mutations. Fifteen *tet*^{s} mutants and six *tet*^{r} colonies were selected and tested for the presence of large plasmid using 'pulse field gel electrophoresis' technique. Four of the *tet*^{s} mutants lacked the large plasmid. However, one of the four mutants had a new smaller 85 kb plasmid. All the six *tet*^{r} colonies tested showed the presence of 255 kb plasmid. The 85 kb plasmid showed homology with the chromosomes of *S. rimosus* and several other strains of *Streptomyces* and *Nocardia*, but not with the 255 kb plasmid. Possible mechanism for the generation of 85 kb plasmid and the possibility of the location of some *tet*^{r} genes on 255 kb plasmid are discussed.

SEVERAL species and strains of *Streptomyces* are now known to harbour giant linear plasmids[1–6]. By and large, the function and significance of these plasmids is not known. However, it has been shown that SCP[1], a giant linear plasmid in *S. coelicolor*, carries the methylenomycin biosynthetic gene cluster[7]. Some of the plasmids such as SCP[1] exist both in an integrated or extra-chromosomal form[8]. We have earlier reported the occurrence and isolation of a 255 kb linear plasmid from *S. rimosus*[9], which is a tetracycline-producing strain. Thus, it seemed of interest to determine if any of the genes connected with tetracycline production are located on the 255 kb plasmid. Antibiotic-producing strains must become resistant to the antibiotic to prevent its own death[10] and the genes for resistance are often linked to the antibiotic production genes or are coordinately regulated[11]. In general, however, genes for antibiotic production and resistance seem to reside on the chromosome. The possibility, nevertheless, remains that some of these genes may be carried on the plasmid. The *S. rimosus* strain used here is tetracycline-resistant. Several tetracycline-sensitive mutants were isolated. The presence of the 255 kb plasmid was examined in these. We report here the observations made on these experiments.

Spores of *S. rimosus* were suitably diluted in distilled water and spread on petri plates containing R5 medium (100 ml containing 10.3 g sucrose, 0.025 g K_2SO_4, 1.0 g $MgCl_2 . 6H_2O$, 1.0 g glucose, 0.01 g Difco casaminoacids, 0.2 ml trace elements, 0.5 g Difco yeast extract, 0.1 ml of 0.5% KH_2PO_4, 0.4 ml of 5 M $CaCl_2 . 2H_2O$ and 0.7 ml of 1N NaOH). The plates were then exposed to ultraviolet radiation from a 15 Watt germicidal UV lamp (Philips) for 45 s at the rate of 13J/m^2/s. The UV irradiation resulted in approximately 99% killing. The plates were then incubated at 30°C for 2 days and the colonies which appeared were tested for tetracycline resistance at 10 µg/ml by replica plating in Difco nutrient agar plates. The sensitive cultures were grown for 2 days at 30°C in a water bath shaker with good aeration by incubating 105 spores in 50 ml yeast extract–malt extract (Difco) medium containing in 100 ml, 0.3 g yeast extract, 1.0 g glucose, 34 g sucrose, 0.2 ml of 2.5 M $MgCl_2$ and 2.5 ml of 20% glycine. The mycelium was harvested by centrifugation and washed twice with P buffer (10.3 g sucrose, 0.25 g K_2SO_4, 10 ml of 3.68% $CaCl_2 . 2H_2O$ and 1.6 ml of 0.1 M Tris HCl in 100 ml pH 8.2). A total of 4,340 replica-plated colonies were examined for tetracycline sensitivity. Out of these, 15 were found to be sensitive to 5 µg/ml or less of tetracycline, yielding a mutation frequency of ~0.34%. In contrast to this the spontaneous frequency of *tet*^{r} *tet*^{s} was estimated at about 0.1%. Further characterization of the 15 isolates was done to determine the antibiotic concentration at which these were sensitive (Table 1). DNA samples from the 15 tet-sensitive isolates and 6 tet-resistant control colonies were examined for the presence of the 255 kb linear plasmid by pulsed field gel electrophoresis as described earlier[9]. All 6 *tet*-resistant DNA samples showed the presence of 255 kb plasmid. Out of 15 *tet*-sensitive DNA samples the 255 kb plasmid could not be detected in 4. In one of the 4 cases, a new smaller plasmid was detected instead. This, too was characterized as a linear plasmid with an estimated size of 85 kb (Figure 1). The 255 kb plasmid was found

Table 1. Antibiotic sensitivity of the 15 isolates

Isolate name	Inhibitory concentrations of tetracycline (µg/ml)	Presence of 255 kb plasmid
MR1	1 0	–*
MR2	1 0	–
MR3	1.0	+
MR4	1 0	–
MR5	1 0	+
MR6	1 0	+
MR7	2 0	–
MR8	2.0	+
MR9	2 0	+
MR10	2 0	+
MR11	2 0	+
MR12	2 0	+
MR13	3 0	+
MR14	3 0	+
MR15	5 0	+

*85 kb plasmid is present in this isolate

This paper is dedicated to the memory of Dr N. K. Notani who expired on 18 March 1994

Figure 1. PFGE analysis of UV irradiated 'transformed' type containing 85 kb linear plasmid (lane 1) and lambda *vir* DNA ladder as standards (lane 2). The electrophoresis was carried out at 5 V/cm with a switching time of 60 s between the two fields.

to be intact in the remaining 11 isolates. The large bright band in lane 1 near the well is presumed to be due to very large DNA fragments of chromosome formed during the sample preparation. A similar band in lane 2 is due to higher order lambda concatemers co-migrating under the conditions used. From these observations a rough correlation emerges. Out of 6 isolates sensitive to 1 µg/ml of tetracycline, 3 had lost the 255 kb plasmid and others had a new 85 kb plasmid. In addition, one isolate that was sensitive to 2 µg/ml tetracycline lacked the 255 kb plasmid. Thus, it would appear that there is one or more tet-resistance gene(s) present on the 255 kb plasmid.

The 85 kb plasmid was electroeluted from agarose gel and labelled with ^{32}P using random primer kit (Boehringer Mannheim). This DNA was used as a probe for Southern hybridization by the method described in reference 12 to test for homology between the linear plasmids. The results are shown in Figure 2. Lanes 1 and 2 show, respectively, DNA samples of wild-type containing the 255 kb plasmid and the UV-irradiated 'mutant' type containing the 85 kb linear plasmid. The corresponding Southern hybridization with probe DNA is shown in lanes 3 and 4. While the probe DNA

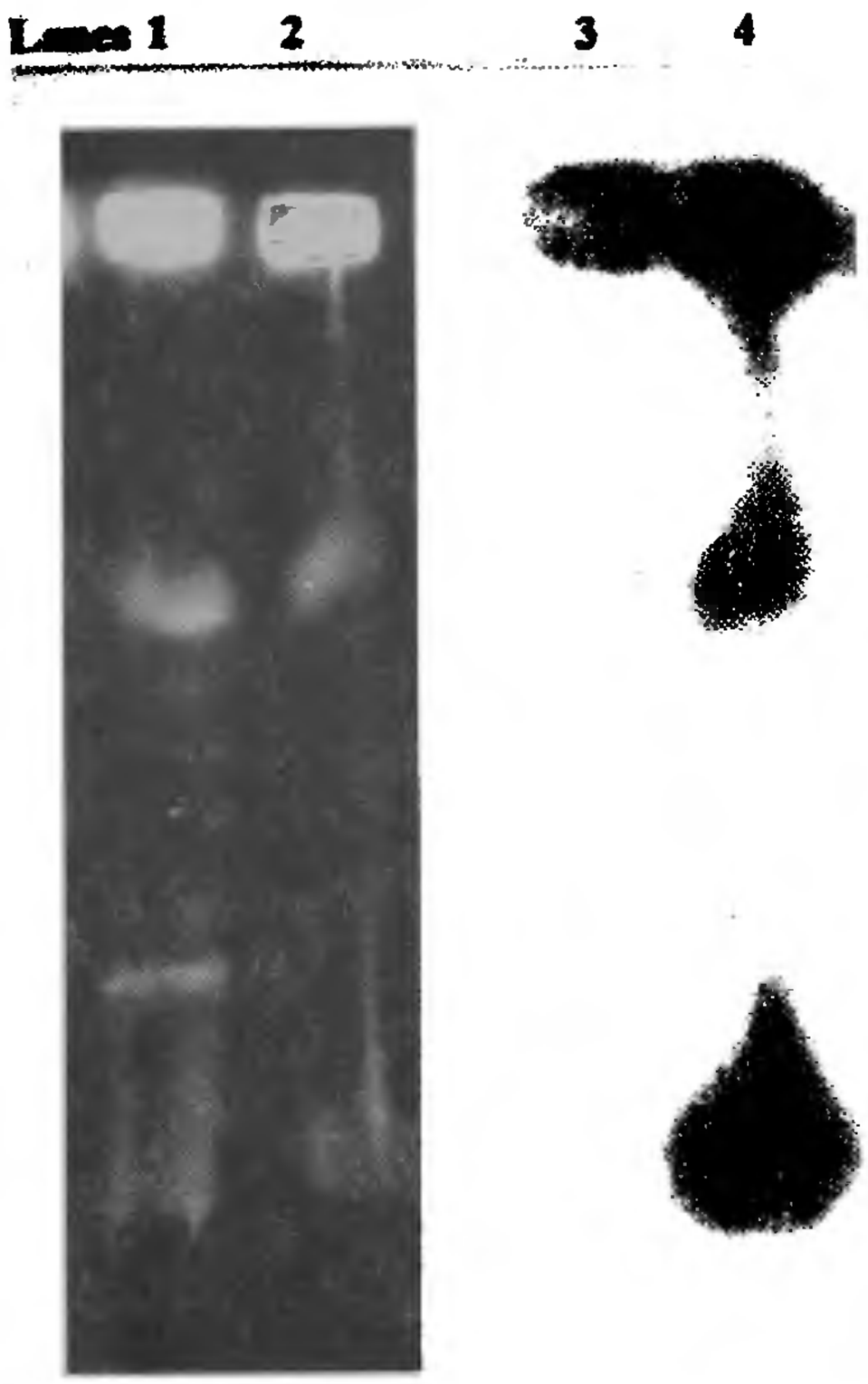

Figure 2. PFGE (lane 1 & 2) and Southern blot (lane 3 & 4) analysis for the linear 85 kb plasmid. Lanes 1 & 3 – *S. rimosus* wild type containing 255 kb linear plasmid; lanes 2 & 4 – UV irradiated 'transformed' type containing 85 kb plasmid. The electrophoresis was carried out at 5 V/cm with a switching time of 120 s between the two fields.

showed strong hybridization with the total chromosomal DNA of both the samples in the wells, it showed no homology with the 255 kb plasmid of wild-type. A repeat experiment to recover the 85 kb plasmid on a second occasion by UV treatment was negative.

The origin of the 85 kb plasmid must remain conjectural. On one hand, it would seem that it has originated from the chromosome; on the other, it has not been possible to isolate it a second time. The possibility of contamination is discounted by a similar sugar requirement profile of the parental strain and the new 85 kb plasmid-containing strain. There seems to be growing evidence that the chromosomes of some of the prokaryotes including certain strains of *Streptomyces* may be linear[13–15]. If that were so for our strain, a simple terminal break could yield a new plasmid. UV irradiation is known to cause such double strand DNA breaks during excision repair of closely opposed pyrimidine dimers[16]. Alternatively, this new plasmid could be 'induced' out of the chromosome by UV.

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Alginate encapsulation technique for indica rice protoplast culture and plant regeneration

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Encapsulation and culture of indica rice protoplasts derived from anther suspension cultures of cv. Rasi in sodium alginate beads was standardized for the first time. Protoplast division (up to 40%) and colony formation (5–6%) were observed without nurse culture. This new technique was superior in terms of division and colony formation compared to agarose entrapment and agarose bead methods. The present protocol would be useful for gene transfer in rice.

PLANT regeneration from protoplasts of japonica rice has been standardized[1-4]. However, protoplast culture and efficient plant regeneration in indica rice are still a major problem and only a few reports are available[5-7]. Use of nurse culture, identification of responsive genotypes and development of suitable embryogenic suspensions for protoplast isolation have been the key to success. Efficient and reproducible protocol for protoplast culture, high frequency colony formation and subsequent regeneration are prerequisites for gene transfer studies. The present study deals with a technique of encapsulation and culture of isolated protoplasts of rice in sodium alginate beads. High plating efficiency in terms of protoplast division, colony formation and plant regeneration was obtained. Regenerated plants were successfully transferred to pots. The protocol can be extended to other cultivars and helps in developing transgenic rice with greater efficiency.

Suspension cultures were established from embryogenic calluses of anthers of indica rice cv. Rasi, a hybrid of T(N)1 × Co29, obtained from Directorate of Rice Research, Rajendranagar, Hyderabad, Andhra Pradesh. High frequency callusing from anthers was obtained on N_6 (ref. 8) medium supplemented with $AgNO_3$. Callus with globular stage embryos was used for establishment of suspension cultures. Suspension cultures were maintained in an amino acid (AA)[9] containing medium supplemented with proline prior to isolation of protoplasts. Four month old suspension culture derived from anther callus was used for protoplast isolation. Embryogenic suspension was obtained on AA medium with 1 g/l proline (AAP) supplemented with 2 mg/l 2,4-D and 0.08 M sucrose. Initial growth of suspensions in N6P medium and transfer of suspension cells to AAP medium prior to protoplast isolation was critical for high yield of stable protoplasts. About 1 g of suspension cells was incubated in 10 ml of an enzyme mixture containing 6% (w/v) cellulase RS, 1.0% (w/v) macerozyme R10 (Yakult Honsha, Japan), 0.5% (w/v) driselase (Sigma, USA), 0.4 M mannitol, 5 mM 2N, *N*-morpholinoethane sulphonic acid (MES) with CPW salts[10], at pH 5.7 (filter sterilized). Protoplasts were purified from the cell debris by passing the protoplast enzyme suspension through 30–45 μ nylon mesh. Protoplasts were pelleted and washed twice in CPW medium with 0.4 M mannitol by centrifugation at 70 *g* for 7 min. Protoplast yield was determined using an haemocytometer. Protoplast viability was estimated using fluorescein diacetate[11].

Freshly isolated protoplasts were cultured by the following three techniques – a) Protoplasts encapsulated in alginate beads; b) Protoplasts encapsulated in agarose beads; c) Protoplasts embedded in agarose.

a) In alginate bead technique, equal volumes of 2.4% (w/v) sodium alginate (BDH, UK) solution with 0.35 M sucrose or 0.4 M mannitol and double strength (DS) N6 PCM (protoplast culture medium) containing protoplasts were mixed properly. Alginate beads were prepared in a solution (50 to 100 mM $CaCl_2$ + 0.4 M mannitol) in a petri dish or a flask. Beads thus formed