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Use of Neomycin for Preferential Selection against *Rhizobium trifolii* in Symbiosis with White Clover (*Trifolium repens*)

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Abstract

Symbiotic parameters were tested on neomycin-containing media with antibiotic-sensitive and -resistant *Rhizobium trifolii* inoculants. Neomycin and kanamycin have similar inhibitory effects on *R. trifolii*, either antibiotic inhibiting growth at concentrations of 50 mg/l. Transposon Tn5 conferred kanamycin and neomycin resistance to *R. trifolii* allowing growth on media supplemented with antibiotic up to concentrations of 400 mg/l. Differential inhibition of nitrate-grown white clover (*Trifolium repens*) plants in an axenic culture system (Petri plates) was observed in terms of dry weight accumulation and visual characteristics. Kanamycin at 50 mg/l and neomycin at 200 mg/l had similar inhibitory effects on plant growth. Symbiotic development by sensitive *R. trifolii* cells was severely inhibited by neomycin at concentrations between 50 and 100 mg/l. Plants nodulated by resistant *R. trifolii* strains maintained control levels of nitrogen fixation with neomycin concentrations up to 150 mg/l. Thus neomycin is proposed to be useful as a selective agent against revertants which have lost transposon Tn5 *in planta* as well as *ex planta*.

Extra keywords: legume, nodulation, genetics.

Introduction

Genetic manipulation has recently been applied to the study of the symbiotic interaction between *Rhizobium trifolii* strains and their plant hosts. The discovery that symbiotic genes controlling nodulation and nitrogen fixation were located on symbiotic plasmids, the mutagenesis of those genes and their subsequent manipulation by cloning and recombination has added much to our understanding of the genetic basis of the root nodule symbiosis (Kondorosi *et al.* 1984). Central to this work is the use of the transposable drug resistance element Tn5, which confers kanamycin and neomycin resistance through the expression of kanamycin kinase (EC 2.7.1.95). This transposon has been successfully introduced into a range of *Rhizobium* species for the isolation of a great variety of mutants (Beringer *et al.* 1978 and many others). When transferred into *R. trifolii*, Tn5 inserts almost randomly at a single site in the genome. Where the insertion occurs into the continuity of a gene, non-leaky polar mutations generally result. The insertion of Tn5 and thus the mutation is linked to the selectable traits of resistance to kanamycin and neomycin (Berg 1977; Kleckner 1977, 1981). This fact allows many types of analyses and *in vitro* manipulations of the bacteria.

A number of Tn5-induced auxotrophic mutants of *R. trifolii* was used to study *Rhizobium–Rhizobium* and *Rhizobium*–plant interactions (Bassam 1982). It was found that the reversion frequencies of the auxotrophic mutants to prototrophy by

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loss of Tn5 were as high as 10^{-7}. Because of this, experiments to determine the symbiotic phenotype of the mutants were often confused by revertants, making it difficult to ascertain the specific symbiotic phenotype of the auxotrophs. The prototrophic revertants were symbiotically effective, producing healthy growth of the plants under nitrogen-free conditions whereas the auxotrophic mutants, in most cases, produced a poor nitrogen-fixing symbiosis with the plant. Furthermore, prototrophic revertants isolated from nodules were shown to be sensitive to kanamycin and neomycin due to the loss of Tn5 (Bassam, unpublished data).

These facts suggest that, if positive selection for Tn5 could be maintained using kanamycin or neomycin during the course of plant tests, the problem of revertants could be minimized, providing, of course, that the antibiotic affected the revertants only and neither the Tn5-induced mutants nor the plants. We describe here an investigation of the potential for such a system of maintaining antibiotic selection *in planta* under axenic conditions.

Materials and Methods

Plants and Bacteria

Certified commercial seed of *Trifolium repens* L. cv. 5826 and *Rhizobium trifolii* strain ANU9000, a spectinomycin-resistant derivative of strain T1 (Skotnicki and Rolfe 1978) were used. Two auxotrophic mutants (ANU9010, pan$^-$; ANU9050 pab$^-$) were derived from strain TA1 (Rolfe *et al.* 1980) by random Tn5 mutagenesis. Auxotrophic strains were resistant to kanamycin (200 mg/l), neomycin (200 mg/l) and spectinomycin (100 mg/l). The presence of the Tn5 sequences was confirmed by Southern hybridization (Bassam 1982; done in collaboration with Dr K. F. Scott, Canberra).

Culture Media

Fahraeus' medium (FM) as described by Rolfe *et al.* (1980) was used in nodulation tests. FM-NO_3 was identical to FM medium except that it contained 10 mM KNO_3 as a nitrogen source. FM-MG was another variant of FM medium designed to support the growth of *R. trifolii* by the addition of mannitol (50 mM) and glutamate (10 mM) as carbon and nitrogen sources. *Rhizobium* growth medium (RGM) 35 (a minimal medium) and RGM36 (a complete medium) were used throughout this study for the culture of *R. trifolii* strains. RGM35 was identical to RGM30A (Mohapatra and Gresshoff 1984) except that 50 mM glucose (filter-sterilized or autoclaved separately) substituted for arabinose and 10 mM KNO_3 was supplied instead of glutamate (Mohapatra and Gresshoff 1984). RGM36 was RGM35 plus 0·1% (w/v) casein hydrolysate and 0·1% (w/v) yeast extract. RGM 36 was the preferred growth medium for culturing the auxotrophic strains and was used in conjunction with RGM35 medium for selective purposes. Purity of cultures was tested on LBG (Miller 1972). Antibiotics and vitamin supplements [i.e. pantothenic acid and *p*-aminobenzoic acid (at 1 mg/l)] were filter-sterilized and added to the medium after it had been autoclaved.

Plant Culture

Plants were cultured on Petri plates 9·9 cm in diameter using the method of Rolfe *et al.* (1980) with minor modifications as outlined by Carroll and Gresshoff (1983).

Biochemical Assay

Nitrogenase activity was measured by acetylene reduction as described for the white clover plate system by Carroll and Gresshoff (1983).

Results

Dose Response of Asymbiotic White Clover Plants to Kanamycin and Neomycin

Plants were grown on FM-NO_3 medium to which various concentrations of kanamycin and neomycin were added. Thirty plants were tested for each concentration of antibiotic and were grown for about 5 weeks without inoculation at which time their dry weights were determined.

White clover plants were much more tolerant to neomycin than kanamycin as shown in Figs 1 and 2. For kanamycin levels of 50 mg/l and over, there was virtually no growth of plants and leaves and roots were stunted and distorted. Fig. 1*a* shows

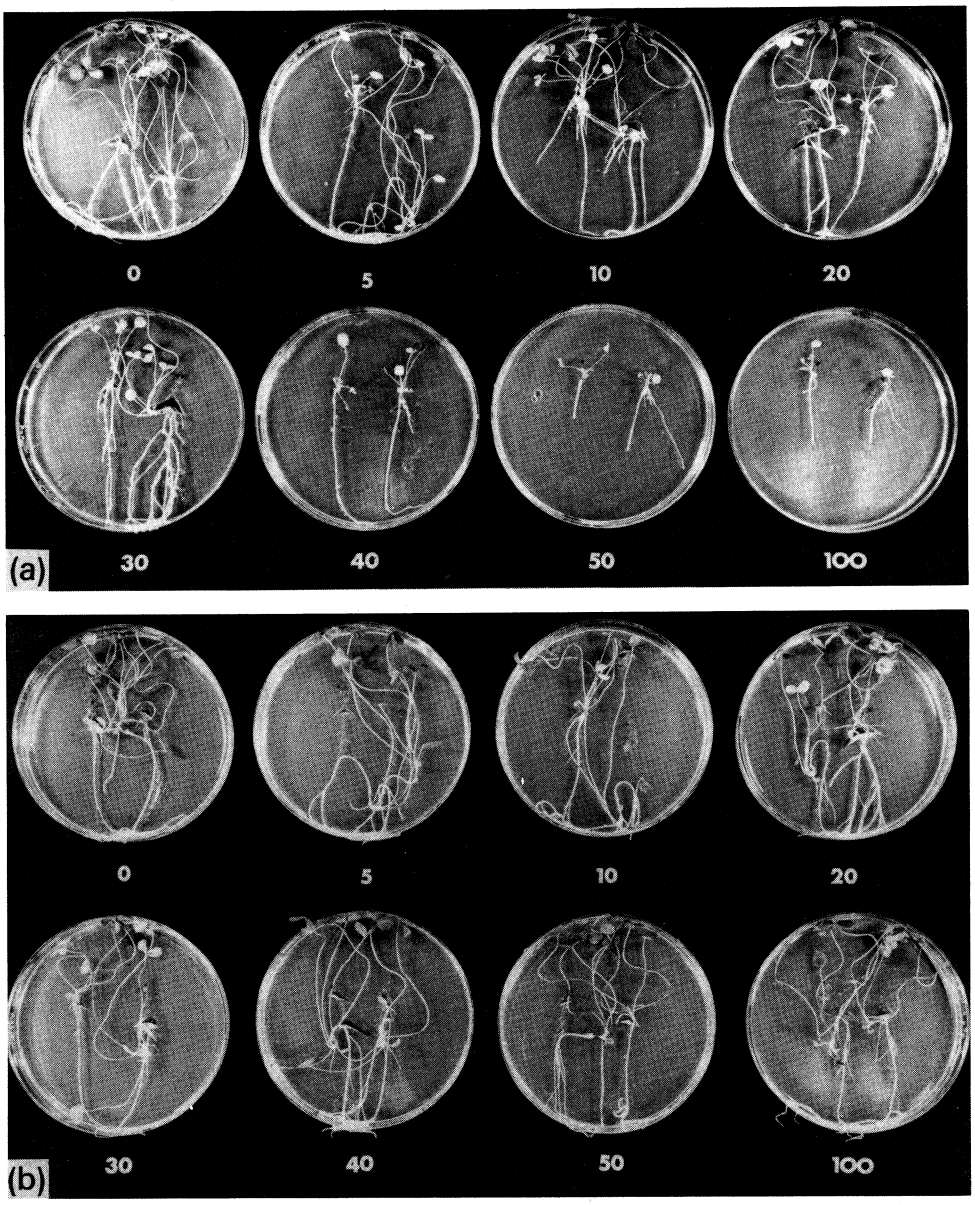

Fig. 1. Effect of various concentrations (mg/l) of kanamycin (*a*) and neomycin (*b*) on the development of white clover plants after 34 days incubation. No inoculum of *R. trifolii* was used; instead NO_3^- (5 mM) was used as a nitrogen source by the plants.

stunted lateral roots (not nodules as may appear from the photograph). Leaves of affected plants remained very small and often had a bright red coloration, indicative of plant stress.

Growth of white clover plants was unaffected by neomycin until concentrations of this antibiotic exceeded 150 mg/l (Fig. 2), but inhibition did not exceed 50% below neomycin concentrations of 250 mg/l. Plants began to show stunted root growth at levels of neomycin higher than 200 mg/l, though the shoots were much less affected. None of the discoloration of the leaves often observed with kanamycin-treated plants was evident for levels of neomycin up to 350 mg/l.

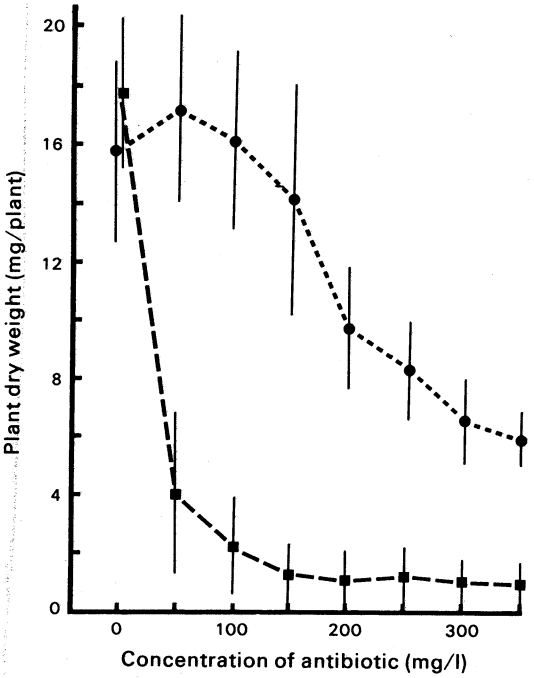

Fig. 2. Effect of different concentrations of kanamycin (■) and neomycin (●) on the growth of white clover plants. Plants were grown for 34 days on FM-NO_3 plates supplemented with various concentrations of each antibiotic. A total of 30 plants (three per plate) were used to obtain data points. Bars indicate standard deviations.

Dose Response of R. trifolii *Strains to Kanamycin and Neomycin*

About 2×10^7 *R. trifolii* cells were normally used to inoculate plants using the plate assay procedure. If the bacteria were applied at this density to FM-MG plates and incubated under standard conditions in a plant growth cabinet, the kanamycin- and neomycin-sensitive control strain ANU9000 failed to grow at concentrations of 50 mg/l of either antibiotic. When 100 times higher inoculum was used, a level of between 150–200 mg/l for kanamycin and 100–150 mg/l for neomycin was required to achieve similar inhibition. However, such inoculant numbers were never used to inoculate plants. Thus, *R. trifolii* strain ANU9000 was slightly more sensitive to neomycin than kanamycin.

The auxotrophic strains (ANU9010 and ANU9050) containing Tn5 and thus expressing resistance to both antibiotics were tested in the same manner on FM-MG medium provided with the particular nutritional requirement of each strain. At 2×10^7 per plate each strain grew at concentrations of neomycin of 350–400 mg/l and of kanamycin 400–500 mg/l.

Effect of Kanamycin and Neomycin on Plants Nodulated with an Antibiotic-sensitive Strain of R. trifolii

Plants were inoculated with the wild-type kanamycin- and neomycin-sensitive strain ANU9000 at 2×10^7 cells per plate. Various concentrations of each antibiotic were

added to the FM medium. After 5 weeks the plants were harvested (20 plants per treatment) and measured for dry weight, nodulation and nitrogenase activity (by acetylene reduction).

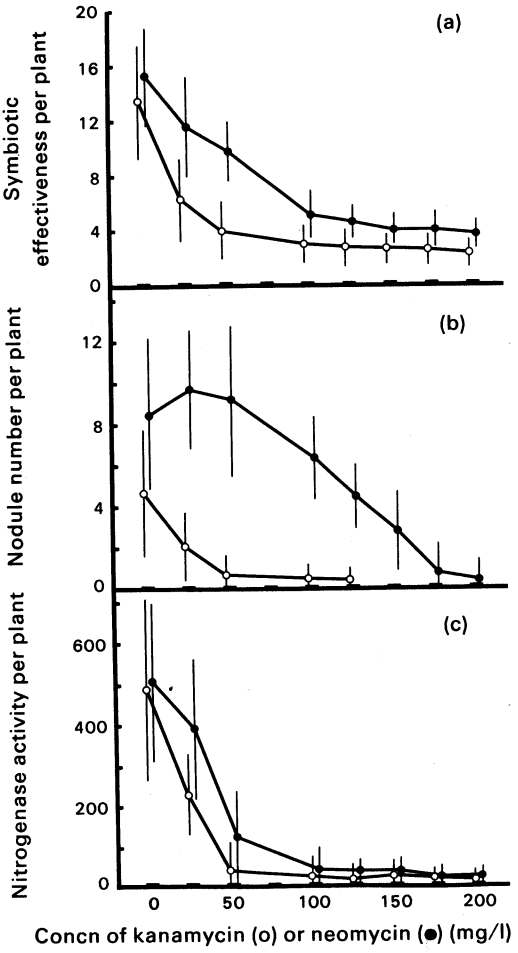

Fig. 3. White clover plants inoculated with the kanamycin- and neomycin-sensitive strain ANU9000. Plants were grown for 33 days with various initial concentrations of each antibiotic: (*a*) symbiotic effectiveness, measured as plant dry weight (mg); (*b*) nodulation, measured as nodule number; (*c*) nitrogenase activity of nodules measured as acetylene reduction (nanomoles ethylene produced per hour per plant). Number of plants ranged between 22 and 27. Bars indicate standard deviations.

In the presence of either antibiotic, each of the measured symbiotic parameters decreased with increasing doses of the antibiotic. Increase in plant dry weight was used as an overall measurement of the health of plants and hence the effectiveness of the symbiosis since, if nodules were not produced or if they did not reduce atmospheric nitrogen, the plants were deprived of a nitrogen source and could not grow normally. The results (Fig. 3*a*) showed that the plant dry weights were reduced to the level of an uninoculated control at a level of 50 mg/l for kanamycin and at 100 mg/l for neomycin. Plants were still able to nodulate in the presence of either antibiotic (Fig. 3*b*). Nodules produced at low levels of neomycin were not healthy in appearance. Most lacked their normal red coloration, being white and very small, and did not normally fix nitrogen. The results for nitrogenase activity (Fig. 3*c*) showed that, after allowing for endogenous ethylene levels, nitrogenase activity had ceased at a level of 100 mg/l for neomycin and 50 mg/l for kanamycin. This showed that,

although nodulation was still possible in the presence of even higher concentrations of antibiotic, *R. trifolii* was unable to establish a functional symbiosis within the nodule. The antibiotics were thus able to select against the expression of the phenotype of a sensitive *R. trifolii* inoculum both *in planta* as well as *ex planta*.

Effect of Neomycin on Plants Nodulated with Antibiotic-resistant strains of R. trifolii

Plants were grown on FM medium containing neomycin at concentrations of 0, 100 and 150 mg/l. These levels of neomycin were those suggested by earlier experiments to be suitable for routine plant assays since they had no measurable detrimental effect on white clover plants but exerted a strong selection against antibiotic-sensitive *R. trifolii*. Kanamycin was not tested since it had a severe inhibitory effect on normal plant growth and development at concentrations required to select against sensitive *R. trifolii*.

Two auxotrophic strains, mutated with Tn5 and thus neomycin-resistant, were used: ANU9010 requiring pantothenic acid and ANU9050 requiring *p*-aminobenzoic acid for normal growth. The plant growth medium (FM) was also supplemented with the vitamin requirement of each auxotrophic strain since it was known that both these auxotrophic strains produced a healthy symbiosis under supplemented conditions (Bassam 1982). The neomycin-sensitive strain ANU9000 was included as a control. After 4 weeks the plants were harvested and scored for plant dry weight, nodulation and nitrogenase activity.

The neomycin resistance of strains ANU9010 and ANU9050 was reflected in the healthy symbiotic phenotype of plants for each concentration of the antibiotic. For all symbiotic parameters (nodulation and nitrogenase activity) tested the Tn5 containing strains were indistinguishable from control plants nodulated with ANU9000 on neomycin-free medium. Fig. 4 shows the ability of plants grown on antibiotics to accumulate plant dry weight, which is an indirect, but integrating, measure of the ability to fix nitrogen and assimilate it during the culture period on nitrogen-free FM medium. Control plants on neomycin-containing medium produced a very poor symbiosis with strain ANU9000. The measured nitrogenase activity of these plants was negligible (data not presented).

Evidence for selection against neomycin-sensitive *R. trifolii* strains in symbiosis with white clover grown on neomycin was obtained in cultural re-isolates from nodules produced by mixed infections. For example, a leucine-requiring, Tn5 induced auxotroph of strain ANU9000 (Bassam 1982) was inoculated onto white clover seedlings and cultured on FM medium with or without the addition of 150 mg/l neomycin. Re-isolation of *R. trifolii* cells by nodule squashes (after surface sterilization of the nodule in 15% calcium hypochlorite for 5 min, followed by a wash with sterile water) and subsequent plating on complete medium (RGM36) showed that in the absence of neomycin during the symbiotic test, effective nodules (appearing at a frequency of up to 20%) contain prototrophic revertants, of which most had lost their resistance to kanamycin and neomycin. Despite our lack of molecular evidence regarding the presence or absence of the Tn5 transposon in the nodule reisolates, we assume that neomycin sensitivity, coupled with the reversion to prototrophy, represents the loss of the transposon. In contrast, the presence of neomycin (150 mg/l) during the symbiotic test eliminated all (n = 140 plants) prototrophic revertants. We tested 750 single-colony isolates from nodules induced by Tn5 induced

leu$^-$ (leucine-requiring) or pyr$^-$ (pyrimidine-requiring) auxotrophs of *R. trifolii* strain ANU9000 and have not detected one prototrophic revertant. These plants were raised in the presence of 150 mg/l neomycin.

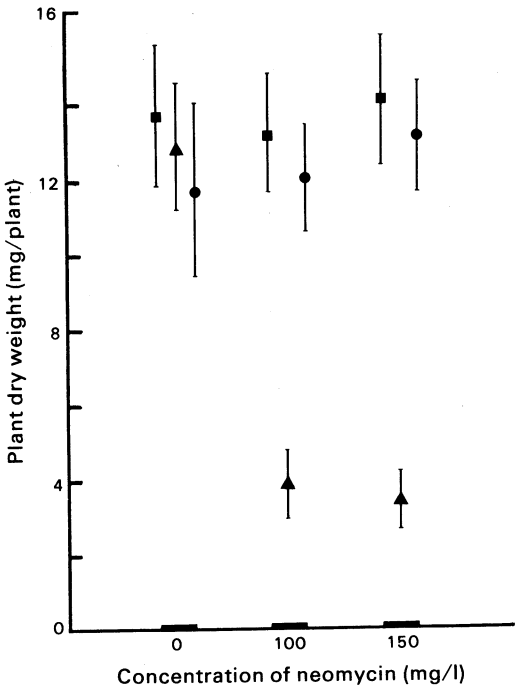

Fig. 4. Effect of neomycin concentration on the symbiotic effectiveness of white clover plants inoculated with neomycin-sensitive [ANU9000 (▲)] and neomycin-resistant [ANU9010 (■), ANU9050 (●)] strains of *R. trifolii* after 4 weeks growth. Number of assayed plants ranged between 20 and 30. Bars indicate standard deviation.

Reconstruction experiments, in which neomycin-sensitive wild-type *R. trifolii* cells were mixed at a 1 : 1 ratio with leu$^-$neo^R cells, showed that in the absence of neomycin, all test plants (n = 12) were symbiotically effective (nod$^+$fix$^+$), while in the presence of neomycin (n = 12) all plants were symbiotically defective (nod$^+$ fix$^-$). Nodule reisolates from such plants showed only a very low (less than 0·1%) presence of wild-type inoculum cells and, at present, we cannot rule out whether these were not extracellular or survivors from the surface sterilization. In any case, such high initial levels of wild-type cells are improbable during a normal symbiotic test. The accumulated data lead us to conclude that neomycin not only restricted nitrogenase activity and resultant plant growth on nitrogen-free medium by white clover plants, but also selected strongly against the presence of neomycin-sensitive cells within the nodule environment.

Discussion

The results presented here show that neomycin may be used to select against sensitive *R. trifolii* strains in symbiotic culture with white clover plants over the range of 100–150 mg/l in the Petri plate assay system. Clearly other concentrations of antibiotic would be required if other culture systems, plant hosts of *Rhizobium* species or strains, were used. At such levels the plants were not measurably affected by neomycin and sensitive *R. trifolii* were completely absent in nodule re-isolates from plants grown on selective levels of neomycin. Though plants infected with neomycin-sensitive *R. trifolii* did occasionally form poor nodules in the presence of the antibiotic, nitrogen fixation did not occur. Thus neomycin was able to act *in planta* as well

as *ex planta* making it very effective as a selective agent when used in conjunction with the axenic plant-assay methods. Infection of plants with *R. trifolii* strains carrying Tn5 resulted in a normal effective symbiosis on neomycin-containing media. Since Tn5 mutagenesis of *R. trifolii* is now widely used, the technique described here should be useful for a wide range of applications.

There was a surprising difference between the effects of neomycin and kanamycin on white clover plants. Whereas plants were very tolerant of neomycin, they were much less so to kanamycin. Even at 50 mg/l (Fig. 2) kanamycin was severely inhibiting normal growth and development of white clover plants. The reason(s) for the difference is (are) unknown. The roots of kanamycin-treated plants seemed most affected; they became very stunted and the formation of laterals was inhibited or abnormal. The antibiotic-inhibited *R. trifolii* was still capable of nodule induction, suggesting that the 'trigger' required for the initiation of the ontogenic sequence persisted or that the trigger was only required at an early stage.

We have used the method described in the text with other auxotrophic Tn5 mutants (leu^-, $methionine^-$ and $pyrimidine^-$) in which variable symbiotic characteristics were observed in the absence of the antibiotic, but not in its presence. Variable (usually symbiotically efficient) plants which were found in the absence of neomycin were routinely found to contain revertant, prototrophic *R. trifolii*, which were neomycin-sensitive.

The procedure may not be absolute because it is possible that a transposon may not be deleted, but rather transposed, to a new location in the genome, resulting in a symbiotic revertant but not in neomycin or kanamycin sensitivity. In our hands with the described strains we find that the latter occurrence is relatively rare (approx. 10^{-9}) as indicated by separate reversion studies (Bassam 1982).

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