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A Cell Line of *Nicotiana sylvestris* with Resistance to Kanamycin and Streptomycin

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Summary. Cell lines resistant to 50 μg ml^{-1} kanamycin sulphate were isolated from cell suspension cultures initiated from a haploid *Nicotiana sylvestris* plant. One line, KR103, has been studied in detail. Resistance of this line was shown to be stable in the absence of the drug. KR103 was found also to be resistant to streptomycin, another inhibitor of 70S ribosomal protein synthesis.

Both KR103 and the sensitive line convert kanamycin, but not streptomycin, to a form which is no longer effective in a bacterial bioassay, while maintaining its toxicity for sensitive plant cells.

KR103 is defective in morphogenesis and plastid development.

Introduction

The attractions of the cell culture system, for the selection of mutants suitable for studies in various branches of plant science, have been discussed extensively in recent years (Melchers, 1972; Street, 1975). Much effort has been directed towards obtaining a range of metabolic and physiological mutants, and the progress has been sufficiently rapid to prompt a number of recent reviews (Maliga, 1976; Maliga et al., 1976; Widholm, 1977)

Among the antimetabolites which have been used as selective agents are two antibiotics, streptomycin (Binding et al., 1970; Binding, 1972; Maliga et al., 1973, 1975; Umiel and Goldner, 1976) an inhibitor of 70S, and cycloheximide (Maliga et al., 1976; Sung, 1976) an inhibitor of 80S, ribosomal protein synthesis. The present report results from a plan to obtain cell lines resistant to a range of other antibiotics, which might prove of value both in studies on the genetics of cytoplasmic organelles, and in the development of selection systems for somatic hybrids. Here we describe the selection of a cell line resistant to kanamycin, which also shows resistance to streptomycin, another inhibitor of 70S ribosomal function.

Nicotiana sylvestris has been chosen as a suitable species for somatic cell genetics as it is a truly diploid member of the *Nicotiana* genus, from which haploid material can be easily obtained (Nitsch and Nitsch, 1969), and which lends itself to a variety of tissue, cell (Dix and Street, 1974), protoplast (Nagy and Maliga, 1976) culture techniques and somatic hybridization by fusion of protoplasts (Melchers, 1976/77).

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Materials and Methods

Plant Material

Haploid plants of *Nicotiana sylvestris* Speg. and Comes were obtained by anther culture according to the method of Nitsch and Nitsch (1969) and were vegetatively propagated by rooting cuttings on their T medium. All the experiments described were performed on material originally obtained from a single haploid plant (SH13).

Culture Conditions

Media used for callus and cell suspension cultures were based on Linsmaier and Skoog's RM medium (1965). Two variations were used. One, designated RMP (Maliga et al., 1977) was used for the routine culture of callus and cell suspensions, and for the isolation, culture, and testing, of resistant lines. The second, designated RMO (Maliga et al., 1973), was used in investigations on morphogenesis, greening, and plastid development. It is a medium which promotes shoot formation in normal cultures of *N. sylvestris*.

Callus cultures were initiated from leaf strips of sterile haploid plants and subcultured at intervals of about 4 weeks. Suspension cultures were commenced by dispersing 0.5 g callus, from the first or second culture passage, in 50 ml liquid medium in 250 ml Erlenmeyer flasks. They were maintained on a rotary shaker (120 rpm) and subcultured, after filtration through 1 mm mesh, every 21 days. In some cases the cell number was determined according to the method of Henshaw et al. (1966). Selection for kanamycin resistance was performed during the first suspension passage, following the initiation passage (i.e. 21 days after the commencement of the suspension). All cultures were incubated at 28° C.

Growth tests on callus cultures were performed by transferring small callus pieces (0.03 g) to agar medium (5 ml per penicillin flask) and incubating for a suitable period (28–42 days) before recording their fresh weight. The values given represent the means of ten replicates.

Estimation of Drug Concentrations

The concentrations of kanamycin or streptomycin in inactivation or uptake experiments were assessed by agar gel diffusion assay using *Escherichia coli*, K12. The bacteria were spread on the surface of 25 ml YTB medium (Orosz and Sik, 1970) solidified with 1.5% agar in 10 cm diameter petri dishes, and samples (culture medium or cold water extracts of cells), alongside standard solutions, were added to 0.5 cm diameter wells (0.05 ml per well). The drug concentrations were calculated from the diameters of the inhibitory zones after overnight incubation at 37° C.

Electron Microscopy

Callus pieces were fixed in high molarity Karnovsky's solution (Karnovsky, 1965) for 4 h at 4° C. The samples were then washed with 0.15 M phosphate buffer (pH 7.2) and postfixed with osmium tetroxide as described by Milloning (1961). Samples were dehydrated by passing them through an ethanol series to pure ethanol, and embedded in Durcupan (Fluka). Ultrathin sections were cut on a Porter-Blum MT-1 ultramicrotome. Sections were stained with lead citrate (Reynolds, 1963) and viewed in a JEOL-100B electron microscope.

Results

1. Selection of Kanamycin Resistant Cell Lines

Preliminary tests demonstrated that growth of callus and suspension cultures was almost completely inhibited by concentrations of kanamycin sulphate greater than 20 μg ml^{-1}. Subsequently cell suspensions were inoculated at 10^5 cells ml^{-1} into liquid medium containing 25 or 50 μg ml^{-1} of the drug. After 4 weeks no cell number increase was observed in any of the flasks and the cell aggregates had turned brown or black. After 6 weeks however, 3 out of 15 flasks of the lower kanamycin concentration were found to contain a number of pale cell aggregates of various sizes. The remaining data described in the present paper refer to lines (KR101–KR135) obtained from one flask, and therefore possibly all of the same origin; and particularly to one line (KR103).

Lines KR101–KR135 were tested for kanamycin resistance on solid medium. In the first test ten, and in the second test nine, of them were resistant to

Table 1. Stability of resistance of KR103 to growth inhibition by kanamycin sulphate. Values are mean fresh weight (g) after 35 days. Initial fresh weight was 0.025 g. KR103[1] is KR103 maintained for the previous 4 culture passages on medium containing 50 μg ml^{-1} kanamycin sulphate, KR103[2] is KR103 maintained for the same period in the absence of kanamycin, and SH13 is the sensitive control. S.E.M. is always less than 10% except * where it is 16%

	Kanamycin sulphate (μg ml^{-1})		
	0	25	50
KR103[1]	1.425	1.482	1.229
KR103[2]	1.376	1.352	1.210
SH13	1.380	*0.046	0.030

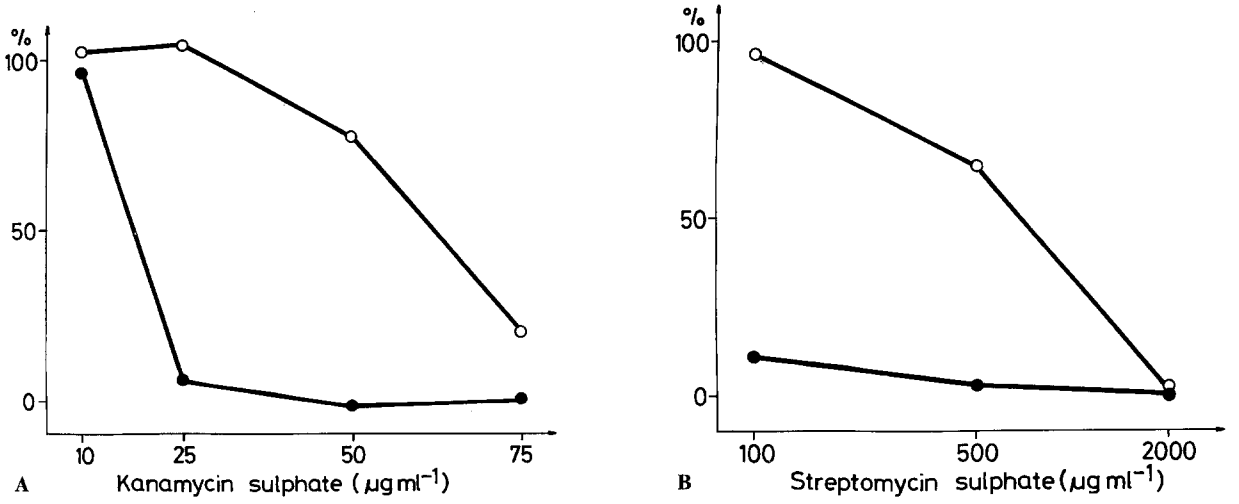

Fig. 1A and B. Test of the KR103 line for kanamycin (**A**) and streptomycin (**B**) resistance. KR103 (○) is compared to a sensitive control, SH13 (●). The values are the increase in fresh weight given as a percentage of that of the same line grown in the absence of any antibiotics. The initial fresh weight was 0.03 g. The final mean fresh weights in the absence of antibiotics were between 1.60 and 1.85 g. The experiments were scored 35 days after inoculation

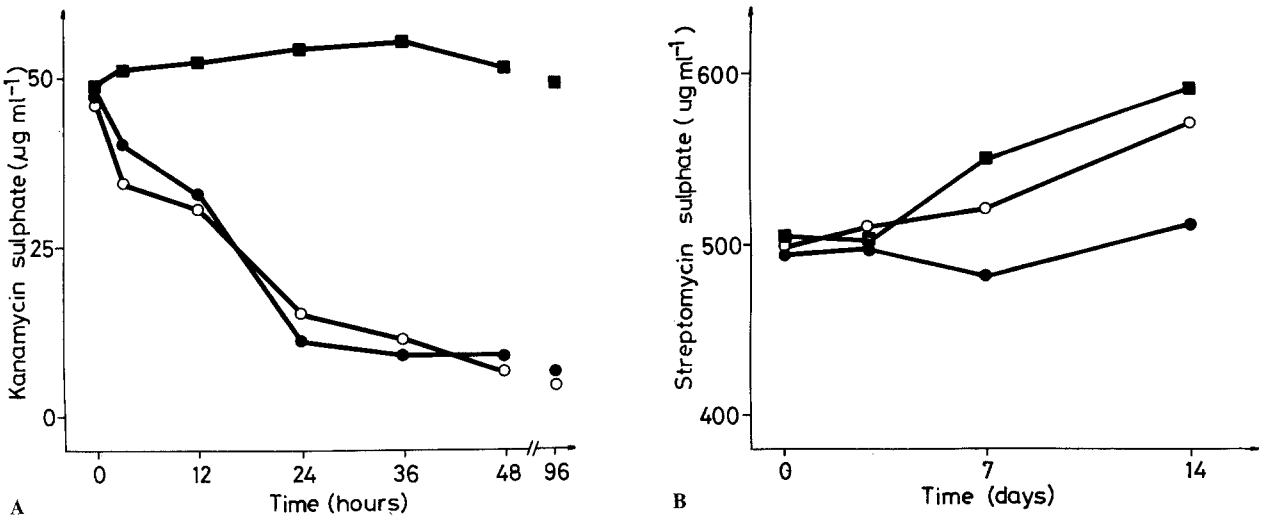

Fig. 2A and B. Effect of cell cultures on kanamycin (**A**) and streptomycin (**B**) in the culture medium. KR103 (○), and a sensitive line, SH13 (●) were each inoculated at 10^5 cells ml^{-1} into liquid medium and cultured alongside uninoculated flasks (■)

kanamycin, compared to control lines. In these early tests, however, there was partial growth inhibition of all lines. This may have been due to segregation since it disappeared in later tests, in which there was no growth inhibition at 25, and only slight inhibition at 50 μg ml^{-1} kanamycin sulphate (data for KR103 are given on Fig. 1A).

Kanamycin resistance, in the case of KR103, was shown to be stable when the line was grown in the absence of the drug (Table 1).

2. *Cross-Resistance to Streptomycin*

Cross-resistance of KR103 to streptomycin, another aminoglycoside antibiotic, was demonstrated in growth tests (Fig. 1B). A similar level of cross-resistance was found in two other lines, KR115 and KR116.

3. *Fate of Antibiotics in the Culture Medium*

A common mechanism for antibiotic resistance in bacteria is conversion of the drug into a biologically inactive form, by one of several means (Benveniste and Davies, 1973). Inactivation of antibiotics in the culture medium, or uptake by the cells, followed by inactivation, would both result in a reduction of the drug concentration in the medium. This pssibility was examined for kanamycin and streptomycin in KR103 and a sensitive cell line, SH13.

Table 2. The growth of KR103 and SH13 callus in spent kanamycin medium. The medium was prepared by incubating cells (10^5 cells ml^{-1}) in medium containing 50 μg ml^{-1} kanamycin sulphate for 7 days, after which the cells were removed by filtration. The values are the increase in fresh weight after 21 days, given as a percentage of that of the same line grown in the absence of kanamycin, in fresh medium. The callus inoculum was 0.15 g added to 15 ml medium in 100 ml Erlenmeyer flasks, and the final fresh weights in the absence of kanamycin were 0.81g (KR103) and 0.66 g (SH13)

	Fresh medium		Spent medium	
	Kanamycin sulphate (μg ml^{-1})		Previous inoculum	
	12	25	KR103	SH13
KR103	74.1	59.4	55.5	48.7
SH13	34.8	28.8	−6.1	3.0

The results for kanamycin inactivation from flasks originally containing 50 μg ml^{-1} kanamycin sulphate are given on Figure 2A. Both resistant and sensitive lines have a similar effect, reducing the bactericidally active kanamycin concentration. The effect was more rapid when 25 μg ml^{-1} was the initial concentration. Within one hour the effective concentration was reduced to 8 μg ml^{-1}. In contrast neither line has an appreciable effect on the streptomycin concentration over a longer time period (Fig. 2B). The concentrations of the drugs inside the cells were difficult to assess accurately, due to the uncertain proportion of the cell volume which can be regarded as a solvent, but estimations based on cold water extracts suggest

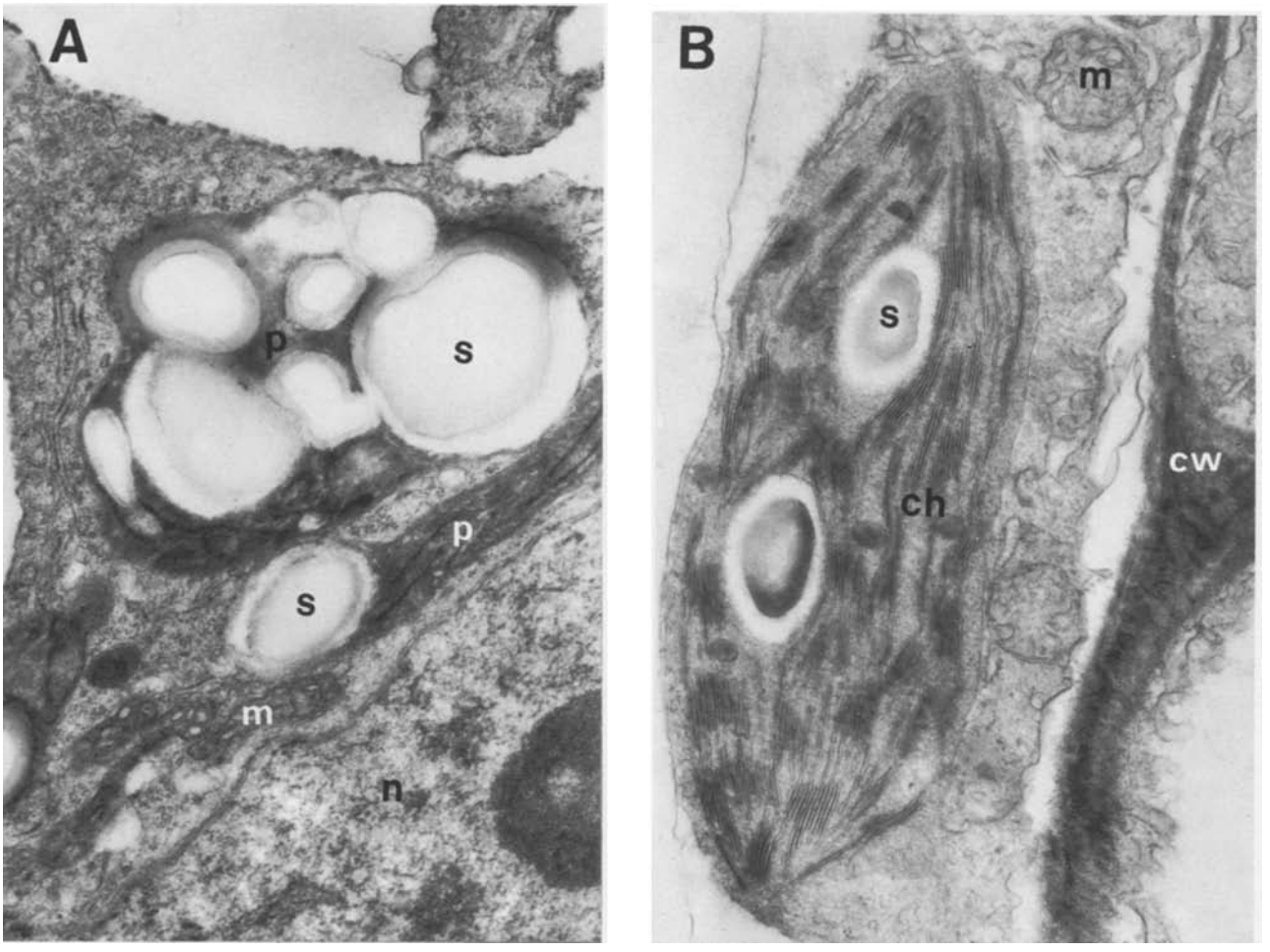

Fig. 3A and B. Plastids of KR103 (**A**) and SH13 (**B**) callus growing on RMO medium. *ch* chloroplast, *p* proplastid, *s* starch grain, *m* mitochondrion, *n* nucleus, *cw* cell wall (×20,000)

that both antibiotics could enter the cells, but were not built up, in the case of either resistant or sensitive lines (results not shown).

To check for possible interference due to infection by microorganisms, samples were removed from the experimental flasks, spread on the surface of nutrient agar medium (YTB) and incubated at 37° C. No contaminating microorganisms were found.

Despite the inactivation of kanamycin, as assessed by bacterial bioassay, the growth of sensitive cells is still inhibited. Either the cells are affected very rapidly by kanamycin, or a kanamycin derivative remains toxic for the plant cells. To examine this possibility the effect of the spent medium on the growth of sensitive and resistant cells was studied. In one experiment, suspension cultures in medium containing 50 μg ml^{-1} kanamycin sulphate were filtered after 7 days, at which time the bactericidally active kanamycin concentration had been reduced to 4 μg ml^{-1}, a level non-inhibitory for sensitive cells. The medium was used to culture callus and suspension cultures of KR103 and SH13 cells. The results, given on Table 2 only for the growth of callus, showed that the growth of sensitive cells was inhibited, while that of KR103 cells was as expected in medium containing kanamycin.

4. Morphogenesis

KR103, like sensitive callus, is unpigmented when grown on RMP medium, and exhibits no shoot or root regeneration. Unlike sensitive callus however, greening and shoot regeneration cannot be induced in KR103 by transfer to RMO medium.

5. Ultrastructure

To determine whether there are any changes in the structure of organelles, which may be related to anti-

biotic resistance, the ultrastructures of KR103 and SH13 cells were compared when each was grown on RMO medium. Characteristic features are shown on Figure 3. Normal chloroplasts are not formed in KR103.

Discussion

Although kanamycin is an aminoglycoside antibiotic, like streptomycin, the method of selection in the present case was very different from that employed by Maliga et al. (1973) for the selection of streptomycin resistant mutants. In that work selection was based on the production of green callus and shoots on morphogenic (RMO) medium, at a concentration of streptomycin which normally prevented greening, but did not completely inhibit the growth of sensitive callus. It is in the nature of this procedure that it favours the selection of lines with modified chloroplasts. The present selection however, was performed using a medium (RMP) which suppresses greening and shoot formation, and is based on the potential for proliferation only. These differences in the methodology may have influenced the types of variant which were obtained.

Line KR103 is deficient in plastid development, and shoot formation. These phenomena may be independent from kanamycin resistance, but it should be emphasized that both generally can be induced in sensitive cultures of the same age. We have no evidence that these characteristics of KR103 have a genetic origin.

Antibiotic resistance in bacteria has been studied extensively (Benveniste and Davis, 1973) and resistance is generally due to alteration of cellular components, preventing uptake of the antibiotic or normal interaction with the ribosomes, or to chemical inactivation of the antibiotic.

Our data demonstrate that KR103 does not convert either kanamycin or streptomycin into a nontoxic compound in the cells or culture medium, and that both can be taken up into the cells. Resistance is based neither on inactivation of antibiotics nor on their exclusion from the cells.

The most likely explanations for the double resistance observed in KR103 cells appear to be multiple mutation affecting ribosomes (Kubitschek and Venema, 1976), or single mutation affecting organelle membranes. The data presented here are compatible with both explanations, but the continued toxicity of a bactericidally inactive kanamycin derivative suggests caution should be applied when interpreting the mechanisms of both toxicity, and resistance to the antibiotic in plant cells.

Somatic hybridisation as a technique, has been successfully applied to *Nicotiana tabacum* varieties and *Nicotiana* species (Melchers and Labib, 1974; Melchers and Sacristán, 1977; Smith et al., 1976; Melchers, 1977) and valuable information concerning the inheritance of antibiotic resistance, may be obtained from such studies, using KR103. Already the properties of this line (kanamycin resistance, deficiency in plastid development and shoot regeneration) have allowed its succesful use in somatic hybridisation studies providing information concerning the control of shoot regeneration (Maliga et al., 1977).

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