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Agrocin 84 Sensitivity: A Plasmid Determined Property in *Agrobacterium tumefaciens*

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Summary. It was shown for some oncogenic *Agrobacterium tumefaciens* strains that agrocin 84 sensitivity is determined by the presence of a large closed circular DNA plasmid, called the Ti-plasmid. Whereas wild-type strain C58 is agrocin 84 sensitive, all Ti-plasmid cured derivatives were found to be fully resistant. Moreover all independently isolated agrocin 84 resistant colonies were stably non-oncogenic and plasmid negative. In a growth experiment carried out at 37° C it was shown that the kinetics of appearance of non-oncogenic cells on the one hand and of agrocin 84 resistant cells on the other were identical. The fact that not all oncogenic, plasmid harbouring, *Agrobacterium tumefaciens* strains are sensitive to agrocin 84, points to the possibility that the genes determining agrocin 84 sensitivity are not essential for tumour-inducing ability.

Introduction

Recently we (Zaenen *et al.*, 1974; Van Larebeke *et al.*, 1974) have demonstrated that a large closed circular DNA plasmid (called the Ti-plasmid) present in *Agrobacterium tumefaciens* strains, is essential for the crown-gall tumour-inducing ability of such strains.

In order to study the precise role of this bacterial plasmid in the neoplastic transformation of normal plant cells to crown-gall tumour cells, it is essential to have genetic markers on the plasmid. Kerr and Htay (1974) have shown that some oncogenic *A. tumefaciens* strains are sensitive to a bacteriocin produced by *A. radiobacter* strain 84 and that when such strains are exposed to the agrocin 84, resistant colonies are selected that are no longer oncogenic. It occurred to us that a possible explanation for these observations could be that both agrocin 84 sensitivity and the tumour-inducing ability are functions controlled by genes on the Ti-plasmid. Curing of the plasmid would than result in plasmid-free cells that are both resistant to agrocin 84 and unable to induce crown-gall tumours. In this paper we describe experiments that demonstrate that this hypothesis is correct.

Materials and Methods

a) Bacterial Strains. The oncogenic, bacteriocin-sensitive strains used were: *A. tumefaciens* strain C58 (Hamilton and Fall, 1971), B6S3 a cured derivative from the lysogenic strain

A. tumefaciens B6 (Vervliet *et al.*, 1974) and Kerr 14, an Australian *A. tumefaciens* strain isolated by Kerr (Kerr and Htay, 1974).

The bacteriocinogenic strains used were: *A. radiobacter* S1005 (Kersters and De Ley, 1973), *A. tumefaciens* 396 (Kersters and De Ley, 1973) and *A. radiobacter* 84 (Kerr and Htay, 1974).

b) Media. YEB and PA media were as described previously (Vervliet *et al.*, 1974); minimal medium contained (in g/l): K_2HPO_4: 10.5; KH_2PO_4: 4.5; $(NH_4)_2SO_4$: 1.0; sodium citrate $2H_2O$: 0.5; supplemented with separately sterilised glucose: 2; $MgSO_4\,7H_2O$: 0.2 and vitamin B1: 0.005.

c) Oncogenicity Tests. Were carried out on pea-seedlings as described by Manigault and Kurkdjian (1967).

d) Detection and Electron Microscopic Visualisation of Plasmid DNA. Were as described previously (Zaenen *et al.*, 1974; Van Larebeke *et al.*, 1974).

e) Test for the Production of—and Sensitivity Towards Agrocins. Was performed essentially as described by Stonier (1960). It was found that for agrocin S1005 production and sensitivity tests PA plates, whereas for agrocin 84 minimal medium agar plates had to be used.

f) Isolation of Agrocin-resistant Derivatives. Agar-media containing agrocins were prepared by spreading 10^8 cells of an agrocinogenic strain onto agar plates. After 48 h incubation at 28° C the bacterial lawn was killed with chloroform-vapours. The agar was inverted in the petridish so that a sterile agar-surface became available onto which $\pm 10^8$ agrocin-sensitive cells were spread. After 48 h of growth at 28°, single agrocin-resistant colonies can be detected on these plates.

g) The Appearance of Agrocin Resistant Colonies as a Result of Curing of the Ti-plasmid by Growth at 37°. A log-phase culture of the agrocin-sensitive, oncogenic strain C58, grown in YEB medium at 28°, was diluted into fresh YEB medium to about $5 \cdot 10^6$ cells/ml and incubated at 37°. When the titer reached 10^9 cells/ml the culture was again diluted to $5 \cdot 10^6$ cells/ml in fresh medium and further incubated at 37°. At regular intervals samples from the culture were taken, diluted and plated for single colonies on minimal medium agar plates with and without agrocin activity as described in Materials and Methods. These plates were incubated at 28° for 48 h. The proportion of agrocin-resistant colonies in the culture was determined as the ratio of the number of colonies on the agrocin-containing plates to the number on the agrocin-free plates.

Results

Agrocin 84 Sensitivity of Isogenic Strains with and without the Ti-plasmid

As described previously (Van Larebeke *et al.*, 1974) the oncogenic, *A. tumefaciens* strain C58, can be cured of its Ti-plasmid by growth at 37°. Using the method described under e. we have shown that strain C58 is sensitive to the agrocin from the *A. radiobacter* strain 84. Ten independently isolated, Ti-plasmid cured, C58-c strains were also tested for agrocin 84 sensitivity. All ten were found to be fully resistant.

Lack of Oncogenicity and Absence of a Ti-plasmid in Independently Isolated Agrocin 84-resistant Colonies Derived from Strain C58

Agrocin 84 resistant colonies were isolated from strain C58 as described under f. Twenty independent colonies were tested for oncogenicity on pea-seedlings and all were found to be stably non-oncogenic. Furthermore five of these strains chosen at random were tested for the presence or absence of a large Ti-plasmid and all five were found to be plasmid negative.

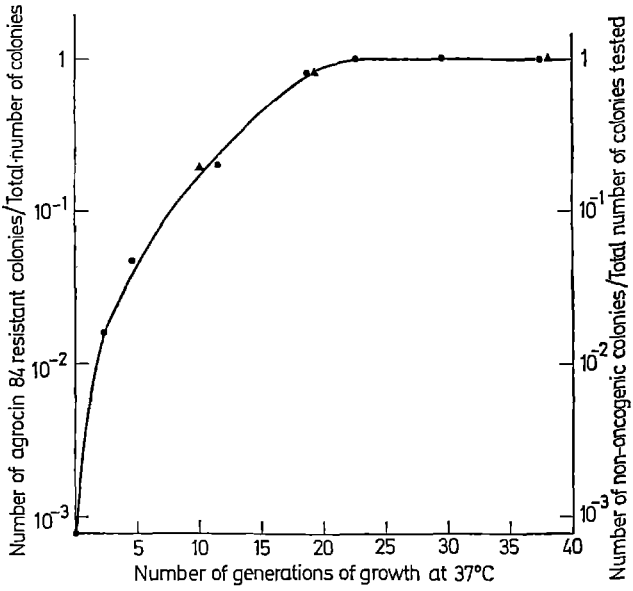

Fig. 1. Appearance of agrocin 84-resistant (—o—) and of non-oncogenic (—▵—) derivatives in cultures of strain C58 grown at 37° C. For methods used see Materials and Methods. After 2 and 4 generations less than 1/10 non-oncogenic colonies were observed

Correlation between Ti-plasmid Curing and Loss of Agrocin 84 Sensitivity

In view of the possibility that Ti-plasmid curing and loss of agrocin-84 sensitivity would be two independent events it was essential to investigate whether or not both events were correlated. To do this we measured the kinetics of appearance of agrocin 84-resistant colonies in a culture of the oncogenic, agrocin 84-sensitive strain C58 grown at 37°, as described under g. and compared these with the kinetics of appearance of non-oncogenic, plasmid free colonies, under the same conditions. As can be seen from the results illustrated in Fig. 1 both events follow identical kinetics. Furthermore at each point ten to fifteen colonies were tested for oncogenicity and for agrocin 84 sensitivity. All the oncogenic colonies were found to be agrocin 84-sensitive whereas all the non-oncogenic colonies turned out to be resistant. In order to be certain that in all the above mentionned experiments, we were in fact dealing with isogenic derivatives of strain C58 and not with contaminants, we systematically applied a number of identification tests.

Table 1 illustrates the results obtained.

Comparison with the Agrocin S1005

In view of the fact that several oncogenic strains, such as the strains B6S3 and 396, are naturally resistant to the action of the agrocin 84, it was of importance to look for other agrocins with different host ranges. Using the method as described by Stonier (1960) a survey was made for strains producing agrocins towards which strains B6 and 396 would be sensitive. *A. radiobacter* S1005 was thus found to produce an agrocin towards which strain B6S3 is sensitive. Agrocin

Table 1. Properties of *Agrobacterium* strains and their derivatives

Strain	Ability to produce 3-keto-lactose (Bernaerts and De Ley, 1963)	Ability to grow anaerobically on nitrate containing medium (Kersters *et al.*, 1973)	Sensitivity to virulent phages of typing set						Sensitivity to agrocin 84	Oncogenicity	Presence of a large plasmid
			S1	S2	S3	S5	S6	S18			
C58	+	+	−	+	−	+	+	−	+	+	+
C58 cured	+	+	−	+	−	+	+	−	−	−	−
C58 agrocin 84 resistant	+	+	−	+	−	+	+	−	−	−	−
B6S3	+	+	−	−	+	−	−	−	−	+	+

S1005 resistant colonies of strain B6S3 were selected for as described under f. Ten independent isolates were tested for oncogenicity and for the presence of a large Ti-plasmid. All turned out to be fully oncogenic and to harbour the Ti-plasmid.

No agrocins were found, thus far, able to inhibit strain 396. However, we discovered that this strain in fact produces an agrocin with the same host-range as agrocin 84. Furthermore it turned out that agrocin 396 sensitivity is also determined by Ti-plasmid genes, since the same positive correlation between the presence of a Ti-plasmid, oncogenicity and agrocin 396 sensitivity was found as for agrocin 84.

Discussion

Our results confirm and explain the observation by Kerr and Htay (1974) that there is a correlation between agrocin 84 sensitivity and the ability to induce crown-gall tumours in some *Agrobacterium tumefaciens* strains. This correlation is the result of the fact that both the agrocin 84 sensitivity and the tumour-inducing ability are properties controlled by genes located on a large plasmid (the Ti-plasmid) present in all oncogenic *A. tumefaciens* strains.

This conclusion was reached by demonstrating

1. That an oncogenic agrocin 84 sensitive strain (C58) when cured of its Ti-plasmid becomes resistant to the action of the agrocin 84.

2. That when agrocin 84 resistant C58 colonies are selected they invariably have lost simultaneously both their tumour-inducing ability and the Ti-plasmid.

3. That the kinetics of appearance of Ti-plasmid cured cells and of agrocin 84 resistant cells as a result of growth at 37° C of strain C58, are identical.

To the best of our knowledge these are the first published observations indicating that bacteriocin sensitivity can be a plasmid determined property. However, it should be stressed that not all oncogenic, plasmid harbouring *A. tumefaciens* strains are sensitive to agrocin 84. This would indicate that agrocin 84 sensitivity as such is not essential for the tumour-inducing ability of *Agrobacterium*. The correlation can be most likely explained by assuming that some oncogenic strains harbour Ti-plasmids that carry both genes determining agro-

cin 84 sensitivity and genes essential for tumour-induction, whereas plasmids in other strains (such as B6S3) do not carry the genes determining agrocin 84 sensitivity. Furthermore it is evident that there are important differences between various agrocins. Indeed there is no correlation between sensitivity and oncogenicity for strains sensitive to the agrocin produced by strain S1005, since isogenic strains resistant to this agrocin are not cured of their Ti-plasmid and are still fully oncogenic.

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