TRANSFER OF THE TUMOR INDUCING FACTOR IN AGROBACTERIUM TUMEFACIENS

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Summary: The Crown-gall tumor inducing factor in Agrobacterium tumefaciens bacterial strain C-58 may be removed by culture at 36°C and it can be reintroduced by inoculation into tomato tumors previously induced by the virulent strain TT-133-1.

Introduction: It has long been postulated that the induction of Crown-gall tumors in plant wounds by Agrobacterium tumefaciens is caused by a tumor inducing principle from the bacteria (1,2). Following transformation, plant tumor cells grow autonomously (without the bacteria) on a simple mineral salts-sucrose medium (3). Spontaneous loss of tumor inducing ability has frequently been observed in bacterial stock cultures and we previously reported a temperature responsive strain (C-58) which lost virulence by culturing at 36°C (4). We now report the reconversion of these avirulent derivatives back to the virulent state. This was achieved by a slight modification of the method of Kerr (5,6), who found that inoculation of A. radiobacter onto tomato Crown-gall stem tumors caused the bacteria to convert to a virulent or tumor inducing state. Thus for the first time the tumor inducing factor may be removed or reintroduced by a process which could occur frequently in nature. This is of considerable interest in view of the recent report that plasmid factors are associated with virulence in these Agrobacterium strains (7,15).

Methods: The bacteria were grown on nutrient agar (NA) or in

nutrient broth (NB) at room temperature. A polymixin resistant isolate (38-7-5) of the avirulent C-58 derivative 38-7 was obtained from a colony appearing on NA plates containing 30 µg/mℓ polymixin. A kanamycin resistant isolate (TT-133-1) of strain TT-133 was obtained from NA plates containing 30 µg/mℓ kanamycin. The polymixin resistant isolate (38-7-5) was completely inhibited by kanamycin (30 µg/mℓ) and the kanamycin resistant isolate (TT-133-1) was completely inhibited by polymixin (30 µg/mℓ). Both isolates were maintained on NA slants containing 30 µg/mℓ of the appropriate antibiotic. The TT-133 strain has been characterized by Keane et al., (8) and the TT-133-1 isolate can be distinguished from the 38-7-5 isolate by: (a) resistant to kanamycin (b) failure to grow on Stonier's minimal medium (9), (c) a negative ketolactose test (10), and (d) the SDS-polyacrylamide gel electrophoresis pattern (11). The litmus-milk test, as well as malonate (12) and citrate (13) utilization also distinguish these strains.

The reconversion of the avirulent C-58 derived isolate (38-7-5) was achieved in 3 experiments by its inoculation into tomato stem tumors induced by TT-133-1. In the first experiment 6-week-old tomato plants (Lycopersicum esculentum L. var. Marglobe) were inoculated with TT-133-1 cells (2×10^9 cells/mℓ, NB) on the stem by needle puncture. The 38-7-5 cells (2.5×10^9/mℓ, NB) were introduced by needle puncture 18 and 25 days after tumor induction. The second experiment was similar except that 38-7-5 was inoculated only once into 49-day-old tumors. In the third experiment tumors were induced on 35-day-old tomato plants with TT-133-1 and inoculated with 38-7-5, 20 days later. Control treatments consisted of (a) tumor induction with TT-133-1 only, (b) stem puncture with 38-7-5 on plants without tumors, and (c) stem puncture without bacteria.

Eleven to 18 days after the 38-7-5 inoculations, the tumors or

stems were surface sterilized (0.525% sodium hypochlorite plus 0.1% Triton X-100) washed, and ground with sand in a mortar containing 10 mℓ of sterile water. One tenth mℓ of a 10^{-2} dilution of the suspension was placed on NA plates containing either 30 µg/mℓ kanamycin or polymixin. Colonies were picked after 48-72 hours for characterization and virulence tests. Virulence tests were performed on 7-day-old primary bean leaves (var. Long Tendergreen) using a slight modification of the method as described by Lippincott and Heberlein (14). To test for growth on minimal media, one loopfull of bacteria (1 x 10^9/mℓ in NB) was transferred to 20 mℓ of Stonier's liquid medium (125 mℓ Erlenmeyer). The cultures were evaluated for growth after 3 days on a reciprocating shaker.

The ketolactose test (10) was performed on 1% lactose agar plates supplemented with 0.1% yeast extract. The plates were inoculated with 0.1 mℓ from NB cultures (1.2 x 10^9/mℓ) and after 3 days incubation flooded with Benedicts Reagent.

The malonate test (12) was performed with 15 mℓ of liquid medium in 125 mℓ Erlenmeyer flasks. Flasks were inoculated from fresh NA slants and incubated 4 days on the shaker. The citrate slants (13) were also evaluated after 4 days incubation. Both the malonate and citrate media were supplemented with 0.2% glutamic acid and 0.01% yeast extract (8). Ten mℓ of standard litmus milk medium (Difco was placed in screw cap test tubes (2.5 x 15 cm). After inoculation from fresh NA slants the tubes were incubated 10-12 days at an angle to increase aeration.

To examine bacterial proteins, overnight NB cultures were harvested and washed with Tris(hydroxymethyl)aminomethane (Tris) buffer (0.05 M, pH 7.0). Samples (200 µg protein) were lysed and boiled 3 min in 1 mℓ Tris-borate buffer (0.075 M, pH 7.2) containing mercaptoethanol (5%), sodium dodecylsulfate (SDS, 1%), sucrose (20%)

Table 1

Reisolation of Agrobacterium tumefaciens parental strains from tomato stems or tumors by the use of nutrient agar antibiotic plates.[a]

Inoculated With	No. Plants Examined	Kanamycin (30 µg/mℓ) No. Colonies[b]	Kanamycin (30 µg/mℓ) Virulent/ Total Isolated	Polymixin (30 µg/mℓ) No. Colonies[b]	Polymixin (30 µg/mℓ) Virulent/ Total Isolated
TT-133-1	3	135	10/10	0	-
		44	10/10	9[c]	0/9
		78	10/10	0	-
38-7-5	3	0	-	82	0/10
		0	-	24	0/10
		0	-	13	0/10

[a]Control plants from experiment 3. The TT-133-1 induced tumors were examined after 33 days and the 38-7-5 inoculated stems after 12 days. Colonies picked from antibiotic plates were bioassayed on 4-6 bean leaves inoculated with 0.05 mℓ of an NB culture (2-3 x 10^9/mℓ).

[b]Average of two plates.

[c]Atypical yellow colonies found on one plate only which were keto-lactose negative.

and bromphenol blue (0.02%). Fifty µℓ of sample was layered on 10% polyacrylamide gels (5 mm x 7.5 cm tubes). The same Tris-borate buffer (0.1 M plus 0.1% SDS and EDTA) was used for electrophoresis and proteins were stained with Coomassie Blue (11).

Results: Typical results (Table 1) from control treatments where tomato stems were inoculated with either TT-133-1 or 38-7-5 alone show that reisolation of the parental strain on the appropriate antibiotic plate is achieved. No virulent cells of 38-7-5 were found indicating that contact with tomato stem tissue did not restore virulence. The virulence transfer experiments (Table 2) indicate that 5 out of 14 tumors examined contained polymixin resistant virulent cells. Three colonies were picked again from each of four virulent polymixin isolates in Experiment 1 and these were bioassayed for tumor induction on bean leaves. All were found to be virulent. Successful transfer of virulence was achieved in all 3 experiments.

All the polymixin isolates grew on Stonier's medium and gave a positive (yellow) ketolactose test. The citrate and malonate utilization tests were negative for 38-7-5 and all polymixin isolates tested but were positive for TT-133-1 (blue). The 38-7-5 strain and all polymixin isolates gave an alkaline litmus milk reaction (purple) while TT-133-1 gave an acid reaction (red). Gel electrophoresis protein patterns were very similar for 38-7-5 and all polymixin resistant isolates but differed distinctly from those of TT-133-1. Thus it may be concluded that the recovered polymixin resistant virulent cells are very similar to the 38-7-5 avirulent parent, but unlike the TT-133-1 virulent parent.

It was of interest to know if the tumor inducing factor was still temperature sensitive in these virulent isolates. A culture of one such strain (DR-1-P5) was incubated in NB at 36°C for 3 days, and then dilutions plated on NA plates. Out of 20 colonies selected none produced tumors on bean leaves. Thus the factor is still

Table 2

Transfer of virulence from the Kanamycin resistant Agrobacterium tumefaciens strain TT-133-1 to the Polymixin resistant strain 38-7-5 by inoculation of TT-133-1 induced tomato stem tumors with 38-7-5.

Experiment[a]	No. Tumors Examined	No. Virulent/No. Colonies Isolated[b] on Plates Containing	
		Kanamycin	Polymixin
1	2	0/0	0/33
	1	3/3	17/20
2	3	6/6	0/30
	2	6/6	13/20
3	4	40/40	0/40
	2	20/20	6/20

[a] Controls inoculated with TT-133-1 or 38-7-5 alone are not shown, but only the parental types were isolated in each case.

[b] Colonies picked from antibiotic plates were bioassayed for tumor induction on 4-6 bean leaves inoculated with 0.05 mℓ of an NB culture (2-3 x 10^9/mℓ).

temperature sensitive in this strain, and this is, of course, also characteristic of the C-58 strain.

A large plasmid has been detected in several virulent A. tumefaciens strains but not in examined avirulent strains (7). Strain C-58 has now been reported to have the plasmid while its avirulent derivatives do not (15). Since it is now possible to remove, and reintroduce, this tumor inducing factor (plasmid) in strain C-58 it should serve as a very useful tool in studies on the Crown-gall tumor induction mechanism. Perhaps the plasmid is transferred to an organelle in the plant cell, or it could be coding for the tumor inducing RNA detected by Beljanski et al. (16).

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