The Loss of Tumor-Initiating Ability in *Agrobacterium tumefaciens* by Incubation at High Temperature

Avirulent strains of *Agrobacterium tumefaciens* have frequently appeared in laboratory stock cultures, but the nature of this change is not understood. Temporary loss of virulence has also been observed following heating[1,2] or prolonged subculture in a glycine-rich medium[3-5]. A number of UV-induced auxotrophic mutants of *A. tumefaciens* have been isolated[6], which are fully virulent only if supplemented with the required nutrient.

We wish to report the conversion of cells of a highly virulent strain of *A. tumefaciens* (C-58) into stable nutritionally sufficient avirulent cells. To our knowledge, this is the first report of the conversion of virulent cells of *A. tumefaciens* into stable avirulent cells under controlled conditions. The derived avirulent cells consistently fail to initiate tumors on tomato stems, sunflower stems, and pinto bean leaves. These cultures have been maintained from 2 to 4 years without regaining virulence.

Our initial experiments involved the cultivation and weekly transfer of 8 virulent, single colony isolates from the C-58 or Ach strains on nutrient agar slants for 4 weeks at 36 °C or at room temperature. After 4 weeks, cultures (Group I) incubated at 36 °C were unable to induce tumors on pinto bean leaves and sunflower stems.

Avirulent cultures have been isolated from strain C-58 following 36 °C incubation for much shorter time intervals (Group II). An overnight broth culture of a virulent, single colony isolate was streaked on nutrient agar and incubated at 36 °C. After various time intervals (Table I) 8 colonies were removed and each inoculated into nutrient broth and grown overnight at 25 °C on a shaker (Parent or Pt isolate). The cultures were adjusted to a selected concentration (turbidometric readings) by dilution with nutrient broth and applied to carborundum-injured pinto bean leaves[7]. Following inoculation, the plants were kept in a dark high humidity chamber at 25 °C for 72 h. Tumor counts or qualitative estimates of the number of tumors per leaf were made after 7 days.

A decrease in tumor induction was noted in many of the Pt isolates incubated 48 h or longer at 36 °C (Table I). That the reduction in virulence observed for these cultures was due to the number of avirulent cells present in the colony, was verified by plating a number of these parent isolates on nutrient agar, and selecting 8–12 colonies. These progeny (Pg) subcultures (shaken in nutrient broth overnight at 25 °C) were tested on bean leaves. Avirulent subcultures (Pg) were obtained from one of the Pt isolates which had been grown at 36 °C for 48 h (Table II). Progeny subcultures indicated that 72 h or 84 h Pt isolates were a mixture of weakly virulent and avirulent cells, while progeny from 120 h Pt isolates were avirulent.

Since loss of virulence could be due to nutritional deficiencies, growth was tested on 3 minimal media which support the growth of the parent C-58 strain. Liquid shake cultures were used and growth was observed after 24 and 48 h. Group I avirulents (4-week heated) grew only on USCHINSKY's medium[8] (glycerol, ammonium lactate, asparagine and salts), and thus appear to be auxotrophs. Group II avirulents (48 h) grew on both MANASSE's[9] (glutamic acid and salts) and the NITSCH and NITSCH (sucrose, nitrate, and salts) medium[10]. Neither Group II virulents nor avirulents grew on the richer USCHINSKY's medium, thus toxicity may be involved in this case. The growth of Group II isolates was also determined in the bean host. Leaves were inoculated in the usual manner and bacteria washed off 10–15 min after inoculation. 3 leaves were harvested at intervals, rinsed in sterile water, and ground with sand in nutrient broth. The homogenate was diluted and streaked on nutrient agar for plate counts. The results indicated that both virulent and avirulent Group II

Table I. Virulence on bean leaves of selected isolates (Pt) of *Agrobacterium tumefaciens* (strain C-58) following incubation at 36 °C for 36–120 h on nutrient agar[a]

Incubation at 36 °C (h)	Relative virulence[b] of 8 selected isolates (Pt cultures)				
	++++	+++	++	+	0
0	5	3			
36	4	4			
48		1	5	2	
60		2	4	2	
72			5	3	
84				5	3
120					8

[a] Each of the 8 colonies selected for bioassay at each time was grown in nutrient broth shake culture overnight (Pt culture) and 4×10^7 cells applied on each of 6 bean leaves. [b] ++++, 200+ tumors; +++, 100–200 tumors; ++, 50–100 tumors; +, less than 50 tumors per leaf; 0, none, but at 4×10^8 cells per leaf an occasional tumor was observed.

Table II. Virulence on bean leaves of subcultures (Pg) derived from parent isolates (Pt) of *Agrobacterium tumefaciens* (C-58) which had been grown at 36 °C for 48–120 h[a]

Parent isolates (Pt)						
Incubation at 36 °C (h)	Virulence[b]	Number of subcultures (Pg) with relative virulence[b]				
		++++	+++	++	+	0
48	+++		7	1		
48	+++		1	10	1	
48	++			7	2	7
72	+				4	4
84	+				5	4
120	0					9

[a] Progeny subcultures (Pg) were selected from plates streaked with cells from the 36 °C grown parent isolates (Pt). The subcultures (Pg) were grown in nutrient broth shake overnight at 25 °C and bioassayed by inoculating 6–12 leaves with $0{,}4 - 4 \times 10^8$ cells per leaf depending on the expected virulence. [b] Rating same as for Table I.

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isolates grow in an apparent log phase at the about same rate for 28 h, but subsequently the avirulent leveled off at a higher concentration per leaf.

The avirulent Group II cells give the keto sugar test[11] characteristic of many isolates of *Agrobacterium* and have a typical *A. tumefaciens* colony morphology at room temperature. Preliminary observations by electron microscopy indicated no gross differences between room temperature grown Group II avirulent or virulent cells.

Although the growth rates were about the same at 25 °C, the avirulent cultures grew 4–8 times as fast as virulent cultures at 37 °C in nutrient broth shake culture. When grown on nutrient agar plates at 37 °C for 24 h, the larger avirulent colonies are easily distinguished from the pinpoint virulent colonies. This growth characteristic has proven to be a much more sensitive and precise method for the isolation of avirulents. If colonies of strain C-58 are grown at 37 °C for 24 h, the cells washed from the plates, and replated at 37 °C, 2–4% of the cells produce the large colony type avirulents. Preliminary data indicate that many of the colonies grown at 37 °C contain a few avirulent cells. Thus the conversion appears to be a high frequency event. All attempts to restore virulence by incubation of the avirulent cultures with lysates from the virulent cells, isolated DNA from virulent cells, Lv-1 phage DNA[12], or the intact Lv-1 phage have so far failed.

Although our results could be explained by the occurrence of a high frequency mutation, perhaps a more likely possibility is the loss of a virulence factor. In other strains this factor may become integrated more tightly with the bacterial DNA. It may be significant that strain C-58 of *A. tumefaciens* is also one of the most virulent strains we have tested. In any event these strains should be extremely useful in the investigation of virulence and tumor induction[13].

Zusammenfassung. Durch Inkubation bei hohen Temperaturen (37 °C) verliert das *Agrobacterium tumefaciens* C-58 allmählich die Fähigkeit zur Gallen- oder Tumorbildung in Pflanzen.

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Localization of Ions in the Mesophyll Cells of the Succulent Halophyte *Suaeda monoica* Forssk. by X-Ray Microanalysis

Distribution of sodium, chloride, potassium and phosphorus between the cytoplasm and vacuoles of leaf cells of *Suaeda monoica* plants was investigated by X-ray microanalysis. Distribution of sodium and chloride in leaf cells of NaCl-treated plants did not coincide. Sodium tended to concentrate in the cytoplasm while chloride was evenly distributed in both cytoplasm and vacuole. Distribution of potassium in the cells of those plants was similar to that of sodium. Potassium content of vacuoles of leaf cells of plants grown under non-saline conditions was higher than that of the cytoplasm.

It is generally accepted that ions absorbed in excess by plant cells are accumulated inside their vacuoles. Halophytes are no exception in this respect and a considerable accumulation of ions in their vacuoles was frequently reported[1–4]. Moreover, transport of sodium and chloride from the cytoplasm into the vacuoles of such plants was believed to be a major cause for plant halosucculence and a mechanism for diminishing the effects of a high intracytoplasmatic salt content[4–6]. It was also frequently spoken of 'salt' effects denoting a similar transport of sodium and chloride. Since no experimental evidence for such assumptions was available, the problem whether ions remain inside the cytoplasm of the succulent leaf cells, or whether they are neutralized in the vacuoles, was investigated.

Seeds of *Suaeda monoica* Forssk. were germinated in Petri dishes and seedlings grown in water culture on a half-strength Hoagland's solution. Sodium chloride, 50 m*M*, was added to half of the plants. Plants were grown for 4 weeks in a growth-chamber under constant temperature (27 °C) and continuous illumination (12,000 lux). Cotyledons and leaves were then taken for analysis.

Material was mounted on top of a microtome blockholder, embedded in fresh rat brain or in liver slices[7], and frozen within 30 sec in a dry ice acetone mixture (− 70 °C). Blocks were cut in a cryostat and the frozen cross-sections placed on cold (− 20 °C) aluminium plates which had previously been coated with a thin layer of silicone grease. A second plate was loaded on top of the mounted sections. The material was left for a few hours in a deep freezer, transferred to a lyophilizer and dried under vacuum (10^{-2} Torr) overnight. A gradual dehydration of the frozen sections – while placed between 2 metal plates – was found to be essential for keeping the sections flat and intact. Strict care was taken to keep the material deeply frozen throughout the procedure. Vacuum was broken with dry air. Following dehydration, the sections were coated with carbon and the location of Na, K, P and Cl was determined by X-ray microanalysis (Jeolco JXA-3A X-ray microanalyzer). An accelerating voltage of 10 KV was used. Sample current was approximately 6×10^{-8} A. Beam diameter was less than 1 μm. Background level was determined by lowering the spectrometer 1° off the specific Bragg's angle. Results for undamaged cells were reproducable.

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