59

Reprinted from
Proc. Nat. Acad. Sci. USA
Vol. 69, No. 1, pp. 191–195, January 1972

Transformation of *Agrobacterium tumefaciens* into a Non-oncogenic Species by an *Escherichia coli* RNA

MIRKO BELJANSKI, MONIQUE BELJANSKI, PIERRE MANIGAULT, AND PIERRE BOURGAREL

Institut Pasteur, Paris XV, France

Communicated by Irwin Chargaff, November 9, 1971

ABSTRACT **Transforming RNA excreted by showdomycin-resistant *Escherichia coli* induces a persistent, heritable, and spectacular change in *Agrobacterium tumefaciens* B_6, a bacterium that carries the oncogenic principle for tumor induction in plants. Transformants possessing new physiological and biochemical properties have completely or partially lost the capacity for tumor induction. They synthetize new ribosomes whose components are profoundly modified. On the basis of biological and biochemical characteristics, one is inclined to consider the completely transformed *Agrobacterium tumefaciens* as a "new species".**

The production by *Escherichia coli* of several RNA species in which the concentration of purines exceeds that of pyrimidines is promoted by showdomycin (1, 2). Mutants resistant to this antibiotic synthesize altered RNAs that are no longer complementary to DNA (2). In these mutants ribosomal proteins and several enzymes have been shown to be greatly modified (1, 2). Some possible mechanisms have been proposed (2) to account for sudden appearance of altered RNAs in the presence of showdomycin. One of these predicted the existence in *E. coli* of an "RNA episome" having purine nucleotides in excess (2, 3). Further investigations confirmed the existence of such an "RNA episome" that is associated with DNA (3). While in wild-type *E. coli*, this "RNA episome" is apparently not functional and does not dissociate from DNA under normal physiological conditions, it appears to dissociate in the showdomycin resistant (Shor) mutant, since this strain excretes a transforming RNA (3). In brief communications, we have demonstrated the transforming capacity of extracellularly released RNA from *E. coli* Shor and that of the RNA episome from wild-type bacteria (3). In the present report, we describe the transformation of *Agrobacterium tumefaciens* by excreted transforming RNA from *E. coli* Shor, accompanied by drastic alteration of the physiological, biochemical, and oncogenic properties of *A. tumefaciens*.

EXPERIMENTAL AND RESULTS

Isolation and purification of transforming RNA from *E. coli*

Mutants of *E. coli* ML 30 Shor were obtained as described for *E. coli* Hfr H M500 Shor (2). The transforming RNA excreted by this mutant was isolated and purified from growth medium as described (2). Among excreted RNA fractions, only those having S values between 5.5 and 6.5 possess the transforming capacity (Fig. 1). The amount of these fractions represents roughly 10–20% of the total excreted RNA. Their base ratio (G+A/C+U = 1.78–2.0) resembles that found for several endogenous RNA species, a general characteristic of showdomycin-resistant mutants.

Transformation of *A. tumefaciens* by RNA from *E. coli*

The procedure for transformation of wild-type bacteria by transforming RNA from *E. coli*, the quantitative effect of active RNA, the inhibitory action of RNase, and necessary control tests have been described (3). In order to produce transformants of *A. tumefaciens*, strain B_6, which is oncogenic for plants, was grown at 30°C in synthetic medium (4). This strain is homogeneous as determined by repeated cloning. Cells were removed by centrifugation and incubated (2×10^7 cells/ml) at 30°C for 3–4 generations or longer (generation time 6 hr) in fresh synthetic medium supplemented with from 0.2 to 2 μg/ml of RNA excreted by *E. coli* ML 30 Shor (total excreted RNA was used in routine experiments). Transformation was judged to be complete when the rate of growth had increased 2- to 3-fold in the presence of RNA. Such a bacterial population plated on solid medium at different intervals gives colonies easily recognizable by their size, which is at least twice that of the wild type. The proportion of transformants increases progressively in the presence of RNA, and wild-type cells finally disappear from the population. If complete transformation is achieved, the high growth rate of each colony is persistent in liquid medium in the absence of transforming RNA. Prior treatment of RNA with RNase in solution of low ionic strength suppress its transforming potential as already described for *E. coli* (3). If transformation fails, there is no increase in the rate of growth. Partial transformation is possible and leads to transformants

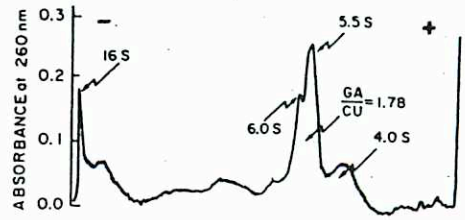

FIG. 1. Densitometer tracing (in the Cary spectrophotometer at 260 nm, 7) of RNAs excreted by *E. coli* ML 30 Shor. RNAs were purified as described (3) and separated by electrophoresis on polyacrylamide gel (4.5%). Length of gel 62 mm. RNA fractions are eluted from sliced gels with SSC (0.15 NaCl–0.015 sodium citrate) at 4°C for 24 hr and dialyzed against distilled water (7); in separate experiments the nucleotides (bacteria were labeled with ^{32}P for 16 hr at 30°C) were analyzed on a Dowex 1 × 2 column, 200–400 mesh.

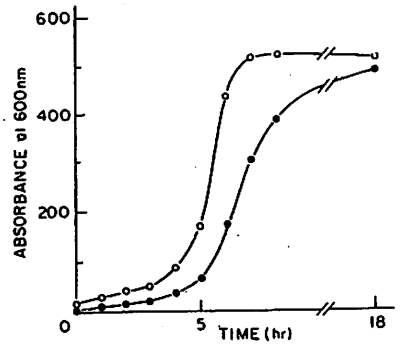

FIG. 2. Growth of *A. tumefaciens* in rich medium (Bacto-tryptone–yeast extract–NaCl, 10:5:5, pH 7.3) at 30°C in the absence of RNAs; wild type (●——●) and B_6-Tr-1 (transformant, O——O). Absorbance at 600 nm in the spectrophotometer Jean et Constant.

with characteristics that are intermediate between those of wild-type and completely transformed cells. Of seven independent experiments, four produced complete transformants (B_6-Tr-1), one gave a partial transformant (B_6-Tr-4) (out of 100 colonies of B_6-Tr-4, one was found to be a second partial transformant and designated B_6-Tr-4-A), and two gave inconclusive results. Spontaneous transformants with the properties described above have not been observed and are not produced when active transforming RNA is replaced by a purine-rich nucleotide mixture or synthetic polyribonucleotides.

Physiological properties of *A. tumefaciens* transformed by *E. coli* RNA

Complete transformants (B_6-Tr-1) are non-oncogenic and grow more rapidly than untransformed cells in rich medium (Fig. 2) and in lactate-containing synthetic medium (4). Both wild-type B_6 and related transformants are strictly aerobic organisms. Neither grows in synthetic medium 63 containing glucose (Table 3), the medium used for *E. coli*. Partial transformants B_6-Tr-4 and B_6-Tr-4-A have similar serological properties as wild-type B_6, while B_6-Tr-1 does not react under the same conditions with anti-B_6 serum (Table 3). Wild-type B_6 and partial transformants give similar results in the test for 3-keto-lactose formation; complete transformants, however, give only a slight positive reaction, which appears with delay. These characteristics are maintained and inherited.

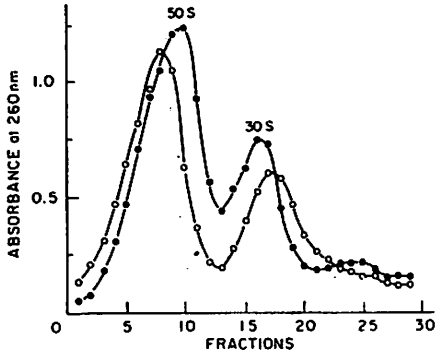

FIG. 3. Profile of *A. tumefaciens* ribosomes after sedimentation in a 5–20% sucrose gradient at 4°C in the Spinco L SW25 rotor at 17,000 rpm for 16 hr as described (7). O——O, B_6-Tr-1 ribosomes; ●——●, control ribosomes.

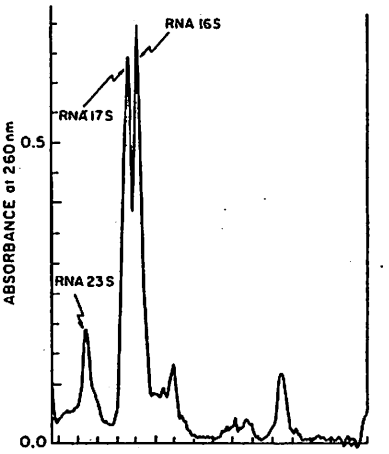

FIG. 4. Densitometer tracings at 260 nm in the Cary spectrophotometer of rRNAs of *A. tumefaciens* B_6 strain. Ribosomal RNAs were isolated by the phenol method from washed ribosomes (bacteria grown in rich medium) and separated by electrophoresis on 3.2% (w/v) gels polyacrylamide as described (8). Length of gels is 62 mm. Amount of rRNA: 7.0–10 μg.

General Characteristics of Ribosomes in A. tumefaciens Transformants. The high rate of growth of complete transformant B_6-Tr-1 (Fig. 2) should be correlated with a change in either the amount of ribosomes or their components, RNAs, or proteins. In fact, transformants have twice the ribosome content of wild-type cells. Ribosomes from wild-type and transformed cells can be distinguished by sucrose gradient analysis (Fig. 3), which shows that the transformation process alters ribosome components. The activity of ribosomes in transformants is excellent, as judged by the rate of growth.

Difference between RNAs of wild-type and transformed *A. tumefaciens*

Figs. 4–7 show the acrylamide gel electrophoresis (3) profiles of RNAs isolated from washed ribosomes of wild-type and transformed cells. The presence of small amount of 23S RNA and of equal amounts of 16S and 17S RNAs are "normal" characteristics of strain B_6 wild-type *A. tumefaciens* (Fig. 4). Electrophoretic profiles of rRNAs of all transformants tested

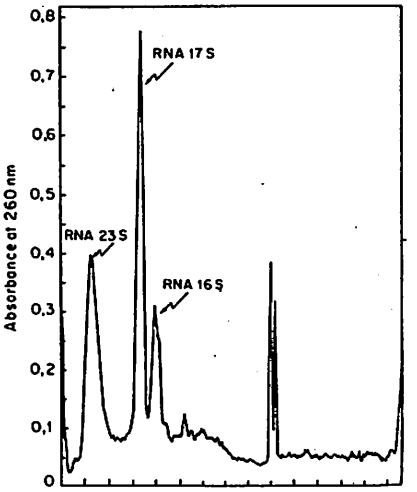

FIG. 5. Densitometer tracing of rRNAs of partial transformants B_6-Tr-4 (see *legend* to Fig. 4).

are quite different from that of the wild-type RNA. The amount of 23S component observed in rRNAs from partial transformant B_6-Tr-4 is substantially higher (Fig. 5) and the amounts of 16S and 17S components have undergone important changes, although both are still present. In the partial transformant B_6-Tr-4-A (Fig. 6) the amount of 23S RNA is about 10-fold greater than that found in wild-type rRNA. Both 16S and 17S components of this transformant are present in smaller amounts than those observed in wild-type cells. In the complete transformant B_6-Tr-1, the amount of 23S RNA (Fig. 7) is 10- to 15-fold that in wild-type rRNA; the 17S component has practically disappeared in this transformant, while a large amount of the 16S rRNA is present. The profiles of RNAs of wild-type and transformed *A. tumefaciens* are clearly different from that of *E. coli* rRNA (2). The nucleotide composition of RNAs in complete transformants (Table 1) shows that the transforming RNA from *E. coli* has imposed on *A. tumefaciens* the synthesis of altered RNAs with an increased purine content.

Different electrophoretic behavior of ribosomal proteins of wild-type and transformed *A. tumefaciens*

70S ribosomal proteins were isolated from washed ribosomes, dialyzed, and then separated by acrylamide gel electrophoresis (2). The densitometer tracings (Figs. 8–10) show that ribosomal proteins of the partial and complete transformants are very different from those of wild-type ribosomes. Thus, certain proteins present in normal ribosomes do not occur in detectable amounts in acrylamide gel analyses of transformant ribosomal proteins, while others are poorly represented; certain proteins are present at the same position in wild-type, as well as in transformed, *A. tumefaciens*. Proteins of strains B_6-Tr-4 (Fig. 9) and B_6-Tr-4-A (Fig. 10) show important qualitative differences from 70S ribosomal proteins of both B_6 wild-type and of complete transformant B_6-Tr-1. On this basis, B_6-Tr-4 and B_6-Tr-4-A constitute two intermediates between B_6 wild strain and complete transformant B_6-Tr-1. It should be particularly noted that there is practically no similarity between the pattern of ribosomal proteins of transformants and that of ribosomes of the *E. coli* mutant ML 30 Shor (Fig. 11) from which transforming RNA is excreted.

TABLE 1. *Base composition of rRNAs of A. tumefaciens wild type and transformant B_6-Tr-1*

	mol per 100 mol of nucleotides			
	Wild type		Transformant	
Nucleotide	23 S	16 S + 17 S	23 S	16 S + 17 S
A	26.0	25.2	30.6	29.3
G	30.4	29.8	33.3	31.4
C	24.7	23.5	19.6	20.6
U	18.9	21.5	16.5	18.7
G+A/C+U	1.27	1.22	1.77	1.56
G+C/A+U	1.20	1.16	1.03	1.08

Base composition of DNA of *A. tumefaciens:* A = 21.7; G = 30.2; C = 28.0; T = 20.1 (6).

Ribosomal RNAs were isolated by the phenol method and separated as described (7). For base-ratio analysis, RNA (1 mg) was hydrolyzed by KOH (0.5 N, 18 hr at 37°C), neutralized, and the nucleotides were analyzed in a Dowex 1 × 2 column, 200–400 mesh (7).

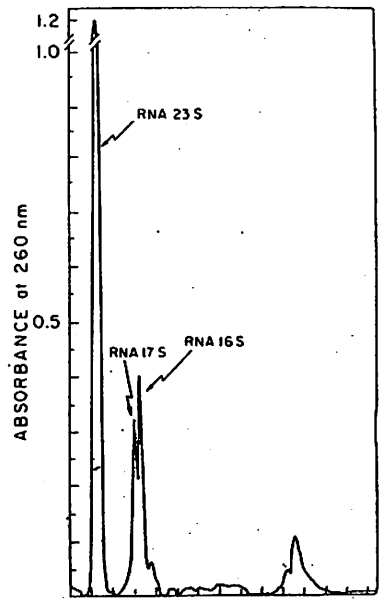

FIG. 6. Densitometer tracings of rRNAs of transformants B_6-Tr-4-A found among the clones of B_6-Tr-4 (one of 100 clones), see *legend* to Fig. 4.

Absence of oncogenic properties in *A. tumefaciens* transformed by RNA from *E. coli*

It is well established that the bacterium *A. tumefaciens* (strain B_6) carries a tumor-inducing principle that causes persistent and heritable changes in the plant host (4, 5). Pea seedlings were inoculated with strain B_6, wild-type *A. tumefaciens* and with the transformants as described (9). Tumors appeared in plants inoculated with the wild-type strain, but

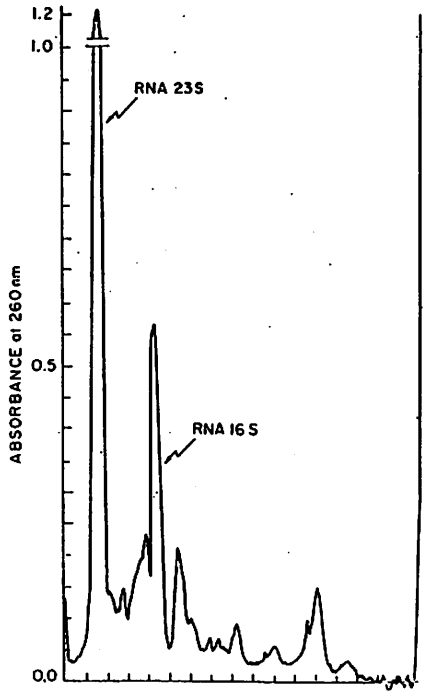

FIG. 7. Densitometer tracings of rRNA of complete transformants B_6-Tr-1 (see *legend* to Fig. 4).

TABLE 3. *Some characteristics of A. tumefaciens strain B_6 and transformants*

	Conditions of growth		Synthetic growth medium			
	Aerobic	Anaerobic	Medium 63*	Medium Stoll†	3-Keto-lactose formation	Serological test
Wild type B_6	+	No	No	+	+++	+++
Transformants B_6-Tr-4	+	No	No	+	+++	++
Transformants B_6-Tr-4-A	+	No	No	+	+++	++
Transformants B_6-Tr-1	+	No	No	+	+ (delayed)	−
E. coli ML 30 Shor	+	+	+	No	No	No

* Synthetic medium 63 routinely used for growth of *E. coli* (3).

† Synthetic medium Stoll (4) is rather specific for *A. tumefaciens*. Test for 3-keto-lactose was performed on bacteria grown for 36 hr at 30°C on one large spot on solid Stoll medium containing 2% of lactose (10). The yellow color characteristic for 3-keto-lactose appears around grown colonies. Serological test was done with the anti-B_6 serum.

process promoted by RNA from *E. coli* resulted in the appearance of partial and complete transformants. The most striking characteristic of complete transformants is their definitive loss of the capacity to be oncogenic in plants, while partial transformants still retain a certain amount of the tumor-inducing principle. The change in the oncogenic properties of transformants seems to be related to the existence of newly synthesized and drastically modified RNAs in these bacteria. Modification of the base composition and of the relative amount of the different RNA species in transformants, as compared to the corresponding components of untransformed cells, is accompanied by qualitative and quantitative modification of ribosomal proteins, increase in cell-growth rate, and appearance of the capacity to synthesize new or modified enzymes (6).

An RNA fraction may be an essential part of the tumor-inducing principle of *A. tumefaciens*, as suggested by Braun and Wood (5). Our previous results showing the genetic potential of transforming RNA and those presented here also suggest that a specific RNA fraction may be involved in infection of plant cells by *A. tumefaciens*. An analogy can be drawn between the process occurring in plant infection by *A. tumefaciens* and that observed with *A. tumefaciens* into which transforming RNA from *E. coli* has penetrated. We have already shown that in *E. coli* the genetic potential of excreted RNA is equivalent to that of episomal RNA associated with DNA in the wild-strain of *E. coli* and in mutant Shor (3). While the RNA episome is not functional in wild-type *E. coli*, it is actively transcribed in mutants (or transformants) that excrete a transforming RNA into the culture medium. The process of excretion of specific RNAs is promoted under given special physiological conditions, at least in bacteria (2, 3). Something similar could take place when a plant is infected with oncogenic *A. tumefaciens*. A specific RNA (episomal RNA for example) may pass from oncogenic bacteria into plant cells and lead to appearance of new biochemical and biological properties that become inherited in these cells.

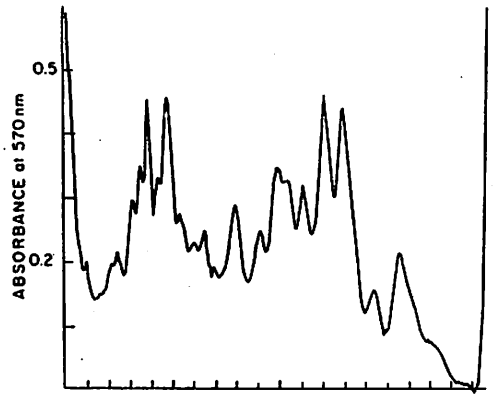

FIG. 11. Densitometer tracings of 70S ribosomal proteins of *E. coli* ML 30 Shor (see *legend* to Fig. 8).

1. Beljanski, M. & Beljanski, M. (1968) *C.R. Acad. Sci. Paris, Ser. D.* 267, 1058–1060.
2. Beljanski, M., Bourgarel, P. & Beljanski, M. (1971) *Proc. Nat. Acad. Sci. USA* 68, 491–495.
3. Beljanski, M., Beljanski, M. & Bourgarel, P. (1971) *C.R. Acad. Sci. Paris Ser. D.* 272, 2107 and 2736–2739.
4. Manigault, P. & Stoll, Ch. (1960) *Phytopathol. Z.* 38, 1–12.
5. Braun, A. C. & Wood, H. N. (1966) *Proc. Nat. Acad. Sci. USA* 56, 1417–1422.
6. Ki Yong, L., Wahl, R. & Barbu, E. (1956) *Ann. Inst. Pasteur, Paris* 91, 212–224.
7. Beljanski, M., Bourgarel, P. & Beljanski, M. (1970) *Ann. Inst. Pasteur, Paris* 118, 253–276.
8. Plawecki, M. & Beljanski, M. (1971) *C.R. Acad. Sci. Paris, Ser. D.* 273, 827–830.
9. Manigault, P. (1970) *Ann. Inst. Pasteur, Paris* 119, 347–359.
10. Bernaerts, M. J. & De Ley, J. (1963) *Nature* 197, 406–408.

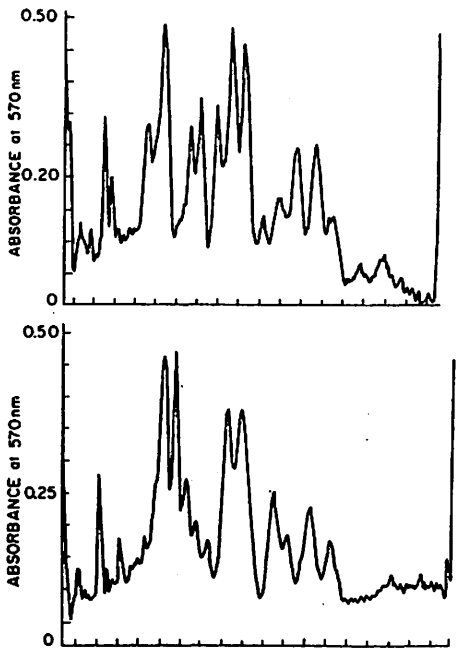

FIG. 8. Densitometer tracings of 70S ribosomal proteins of *A. tumefaciens*. 70S ribosomal proteins were isolated from washed ribosomes and separated by polyacrylamide gel electrophoresis as described (2). Length of gels: 65 mm. Proteins: 80 μg. Densitometer tracings at 570 nm in the Cary spectrophotometer (2) B_6-Tr-1 (complete transformant, *below*); B_6, wild type, *above*.

not with the completely transformed strain, B_6-Tr-1 (Table 2). Clones of this transformant were as inactive as total population. It should be noted that this transformant has lost the capacity to synthesize certain constitutive enzymes and acquired the capacity to synthesize other new enzymes (6). Partially transformed strains B_6-Tr-4 and B_6-Tr-4-A, which on physiological and biochemical bases [moderate increase in rate of growth, rRNAs (Figs. 6 and 7), and 70S ribosomal proteins (Figs. 9 and 10)] are clearly but not completely modified, can be considered as progressive intermediates between wild-type and the complete transformant B_6-Tr-1. The tumor-inducing capacity of B_6-Tr-4 is substantially lower, and that of B_6-Tr-4-A is very much lower, as judged by weight of tumor, than that of the wild type (Table 2). These results indicate that the biochemical changes imposed by transforming RNA of *E. coli* are connected with the tumor-

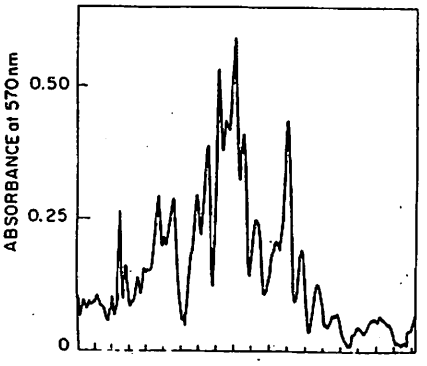

FIG. 9. Densitometer tracings of 70S ribosomal proteins of partial transformants B_6-Tr-4 (see *legend* to Fig. 8).

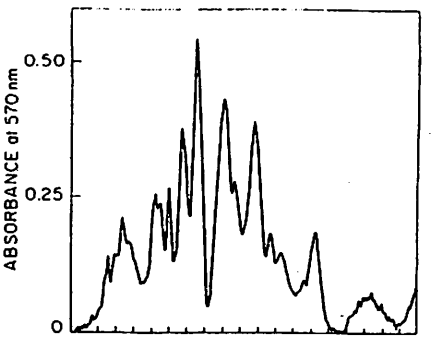

FIG 10. Densitometer tracings of 70S ribosomal proteins of partial transformants B_6-Tr-4-A (see *legend* to Fig. 8).

inducing principle. Attempts to transform *E. coli* into an oncogenic bacterium using RNA from wild-type B_6 have not been successful.

DISCUSSION OF THE RESULTS

The heritable transformation of *A. tumefaciens* strain B_6 into a "new species" by the transforming RNA (or family of RNAs) excreted from showdomycin-resistant *E. coli* is a phenomenon of particular interest. The profound physiological and biochemical changes persisting in partial and complete transformants may be considered as the consequence of "genetic mutation" that is induced by transforming RNA originating from another bacteria species. We suggested elsewhere (3, 8) that the intact and active RNA once introduced into the recipient bacteria may be linked by "RNA ligases" to specific RNAs already associated at specific sites with the DNA of recipient cells. Thus, a large RNA episome could be created, the transcription of which by a particular enzyme (8) would lead to the appearance of modified RNAs, and consequently to synthesis of altered proteins, as demonstrated by important changes observed with ribosomal proteins. In the case of *A. tumefaciens*, the transformation

TABLE 2. *Oncogenic power of A. tumefaciens B_6 wild type, transformants B_6-Tr-1, B_6-Tr-4, and B_6-Tr-4-A in pea seedlings*

	No. of bacteria per wounded host	Confidence limit of the average of adjusted tumor weights (cg) (9)	No. of plants
*B_6 wild type	1.2×10^8	$14.9 < 17.3 < 19.7$	42
B_6-Tr-1	1.2×10^8	No tumors	34
B_6-Tr-4	1.3×10^8	$9.3 < 10.8 < 12.3$	34
†B_6 wild type	At saturation	$26.0 < 28.9 < 31.8$	46
B_6-Tr-1	At saturation	No tumors	37
B_6-Tr-1	(Clones at saturation)	No tumors	100
B_6-Tr-4-A	(Clones at saturation)	$7.1 < 8.0 < 8.9$	45

Confidence interval of 1%. Method of inoculation of bacteria, see ref. 9.

Tumor-inducing capacity of wild-strain B_6 and transformants was routinely tested in several experiments with pea seedlings. The absence of oncogenic properties in the complete transformant B_6-Tr-1 was also observed with *Kalanchoe daigremontiana* and *Datura stramonium*. *, † Two independent series of experiments.