

Isolation of Morphological Mutants of *Agrobacterium tumefaciens*

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Morphological mutants were isolated from a wild strain of *Agrobacterium tumefaciens* at a high frequency by treatment with a nitrosoguanidine. Seventeen of the 20 mutants isolated were temperature-sensitive. At 27 C, the mutant cells were rod-shaped and at 37 C, spherical or branched, whereas the wild-type cells were rod-shaped at both temperatures.

In spite of numerous and brilliant works in molecular biology, the significance of morphology in organisms is still unanswered. Bacterial cells take a defined form (shape and size) under defined surroundings; therefore there must be a control mechanism of morphogenesis.

Rogers et al. (11) reported rod⁻ mutants of *Bacillus subtilis* and *B. licheniformis*, and these mutants were supposed to have an ab-

normal step in biogenesis of cell wall or cell membrane. Formation of branching cells was reported for coryneform bacteria (7) and *Lactobacillus bifidus* (6) under some culture conditions. In this paper, we describe isolation of morphological mutants having a spherical or branching cell shape. The wild type was *Agrobacterium tumefaciens* IAM 1525, which consists of rod-shaped cells and is capable of inducing plant tumors, forming D-glucoside-3-

TABLE 1. Properties of morphological mutants of *Agrobacterium tumefaciens*

Strains of <i>A. tumefaciens</i>	Phenotype marker			Cell morphology		Designated phenotype ^d	
	3-deHase formation ^a	Tumor induction ^b	Resistivity ^c against streptomycin	27 C	37 C	27 C	37 C
F-101, 102, 103, 104, 105, ^e 106, ^e 107, ^e 108, 109, 110, 111, 112, 113	+	+	+	Rod	Spherical	Rod ⁺ Bra ⁻ Fil ⁻	Rod ⁻ Bra ⁻ Fil ⁻
F-201	+	-	+	Rod	Spherical	Rod ⁺ Bra ⁻ Fil ⁻	Rod ⁻ Bra ⁻ Fil ⁻
F-301, 302 ^e	+	+	+	Spherical	Spherical	Rod ⁻ Bra ⁻ Fil ⁻	Rod ⁻ Bra ⁻ Fil ⁻
F-401	+	-	+	Spherical	Spherical ^f	Rod ⁻ Bra ⁻ Fil ⁻	Rod ⁻ Bra ⁻ Fil ⁻
F-501, 502, 503 ^e	+	+	+	Rod	Branched	Rod ⁺ Bra ⁻ Fil ⁻	Rod ⁻ Bra ⁺ Fil ⁻
Wild strain	+	+	+	Rod	Rod	Rod ⁺ Bra ⁻ Fil ⁻	Rod ⁺ Bra ⁻ Fil ⁻

^a Activity of D-glucoside-3-dehydrogenase (3-deHase) (4), which is a specific enzyme in the genus *Agrobacterium*, was examined by 3-ketosucrose-forming ability from sucrose with the cells grown in a synthetic medium of McIntire. Positive (+) means 3-deHase-forming.

^b Plant tumor-inducing ability was examined as follows. The bacterial cells which were harvested from the yeast extract medium at exponential growth phase were inoculated to five positions on a stem of a sunflower plant; and after 6 weeks of cultivation, the numbers of crown galls (tumor bodies) formed at inoculated positions were counted. Positive (+) means formation of five galls (gall formation was observed at all of the inoculated positions), and negative (-) means no gall formation.

^c Positive (+) means resistant against 200 µg of streptomycin/ml.

^d Phenotype abbreviations used are as follows: Rod for rod shaped, Bra for branched, and Fil for filament-shaped; "+" for occurrence of a shape and "-" for the nonoccurrence of that shape. According to this abbreviation, normal-shaped cells are presented as Rod⁺Bra⁻Fil⁻. The ts-morphological mutant cells are either Rod⁻Bra⁻Fil⁻ (spherical) or Rod⁻Bra⁺Fil⁻ (branched) at 37 C. No Rod⁻Bra⁻Fil⁺ mutants were obtained.

^e During maintenance on slants of the yeast extract medium for 1 year, this strain reverted phenotypically to wild type in cell morphology.

^f Cell size is inconstant.

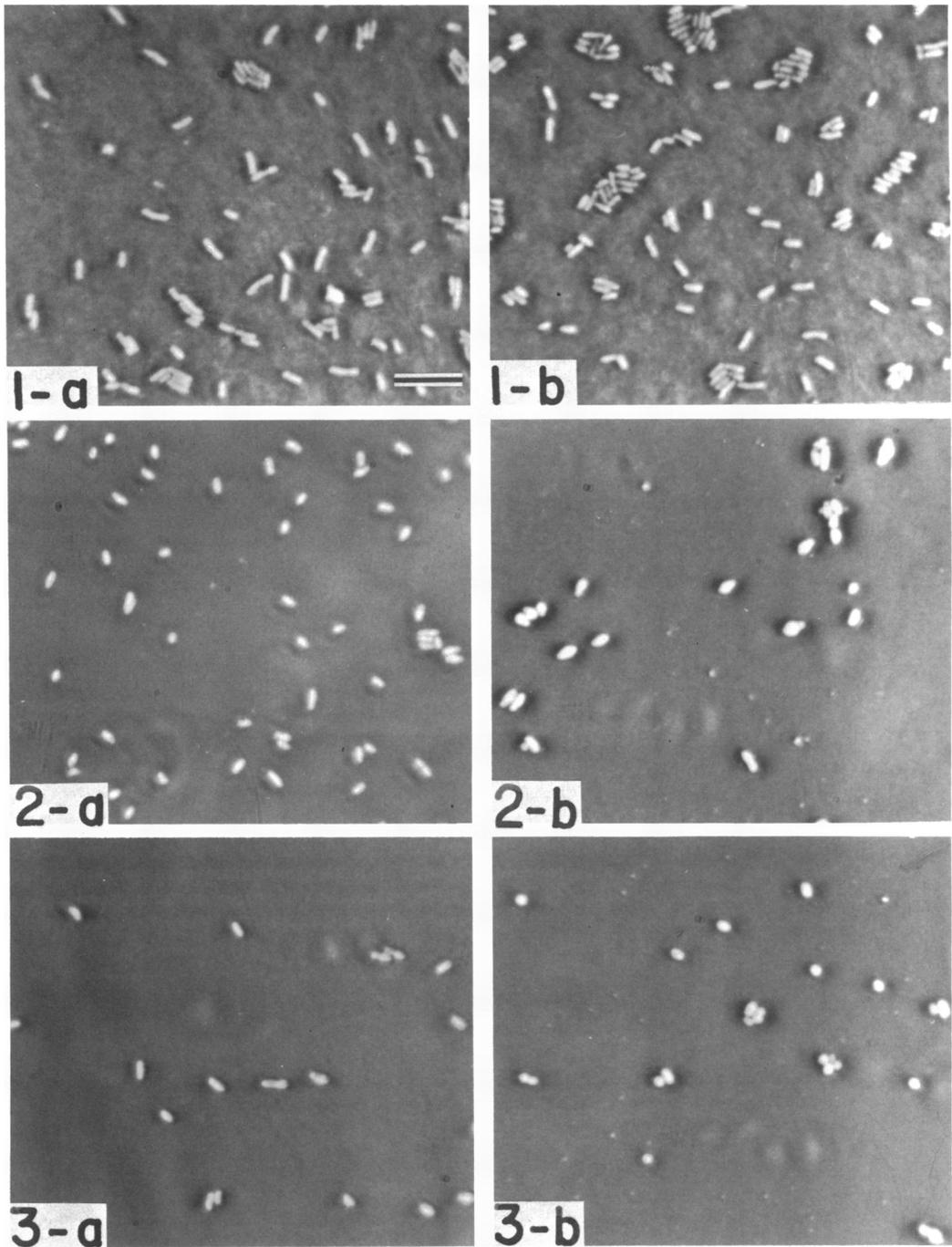


FIG. 1-3. Photomicrographs of morphological mutants of *Agrobacterium tumefaciens*. Preculture was performed in a synthetic medium of McIntire (sucrose-mineral; reference 8) at 27 C with shaking. The bacterial cells at an exponential-growth phase in the preculture mixture were inoculated into the yeast-extract medium (see Text), and cultivation was carried out at 37 C with shaking. Change in cell morphology was followed by observation under a phase-contrast microscope. Micrographs were taken before and after cultivation at 37 C. Photomicrographs 1, 2, and 3 indicate cell forms of F-010 (wild strain), F-108, and F-113, respectively. Presentations as a and b are 0-hr (before) and 4-hr (after) cultivations at 37 C, respectively. Bar, 5 μ m.

dehydrogenase (4), and resisting 200 μg of streptomycin/ml. This is the first report concerning a morphological *ts* mutant with branched cell form in microorganisms belonging to the family *Rhizobiaceae*.

Isolation of mutants was performed as follows. *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG), to give a final concentration of 200 $\mu\text{g}/\text{ml}$, was added to a culture of the wild strain in exponential growth phase in a yeast extract medium composed of 1% yeast extract (Daigo-eiyo Co., Osaka) and 50 mM phosphate buffer (pH 7.0), and cultivation was continued for 30 min at 27 C with shaking. After the NTG treatment, cells were collected on a membrane filter (Millipore filter, pore size 0.45 μm), washed with 50 mM phosphate buffer (pH 7.0), and suspended in a fresh yeast extract medium. Segregation was carried out by incubation of the cell suspension for 3 hr at 27 C

with shaking, followed by plate culture on yeast extract-agar medium at 27 C. Colonies thus formed were replicated on the same agar medium containing streptomycin (200 $\mu\text{g}/\text{ml}$), and incubation was conducted at both 27 and 37 C. Selection of morphological mutants was made by observation of cells in each colony under a phase-contrast microscope.

Twenty mutants were isolated from approximately 2,000 colonies. Morphological properties of the mutants are presented in Table 1 and the figures. Seventeen strains were temperature-sensitive and showed abnormal cell form at 37 C; fourteen strains consisted of spherical cells, and three strains were branched cells in the yeast extract medium. When the temperature was lowered to 27 C from 37 C, the abnormal forms of the mutants returned to the normal, rod form. During maintenance of the mutants on slants of the

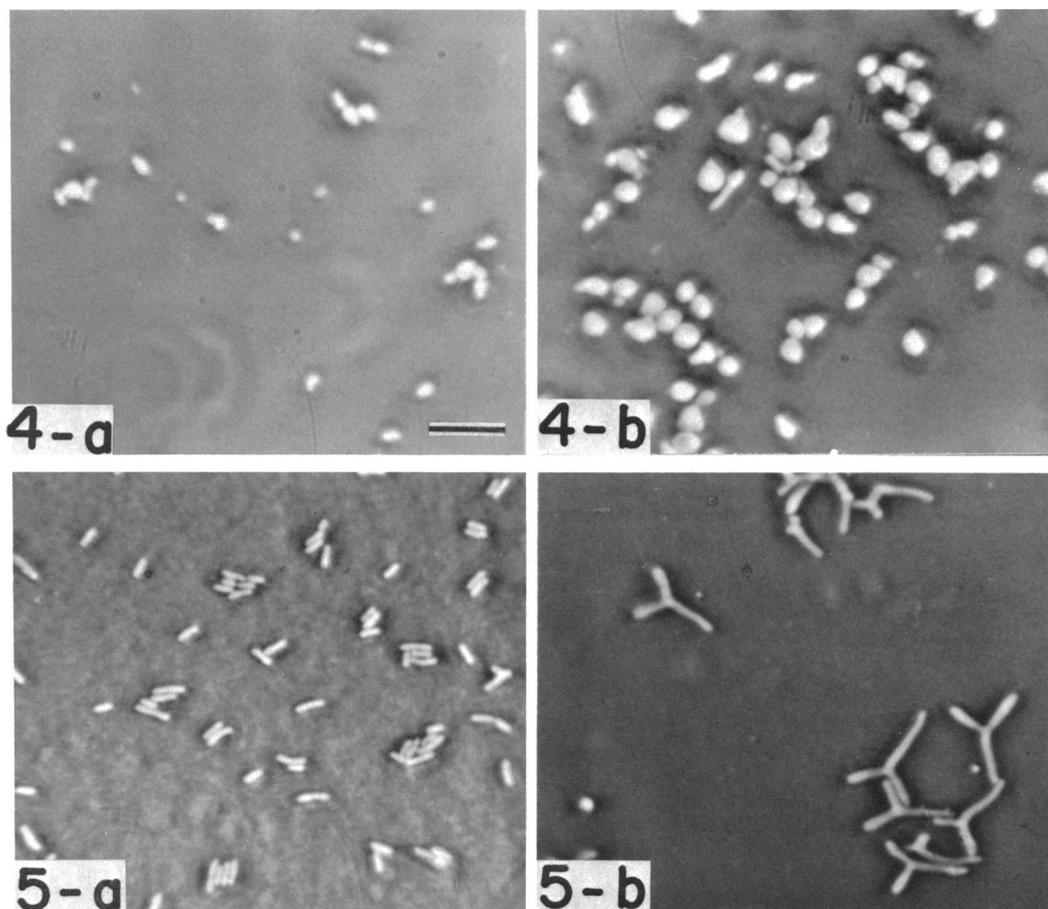


FIG. 4-5. Photomicrographs of morphological mutants of *Agrobacterium tumefaciens*. Preculture conditions are the same as in Fig. 1-3. Photomicrographs 4 and 5 indicate cell forms of F-301 and F-501, respectively. Presentations as a and b are 0-hr (before) and 4-hr (after) cultivations at 37 C, respectively, except 5-b which is a 6-hr cultivation. Bar, 5 μm .

yeast extract medium for 1 year, five strains reverted to wild-type cell shape (Table 1). In this investigation, we isolated no mutants which showed unbranched filamentous cells at either 27 or 37 C.

As morphological mutants of *Escherichia coli*, some strains taking filamentous form in cell shape were obtained by Adler and Hardigree (1), Howard-Flanders et al. (5), and Reeve et al. (10). These were, however, filamentous cells without branching. Tatum et al. (2, 3, 8) isolated colonial mutants of *Neurospora crassa* and *N. sitophila* and demonstrated that these mutant strains are induced by point mutation at the structural gene of an enzyme in sugar metabolism, such as glucose-6-phosphate dehydrogenase or phosphoglucomutase.

As described above, morphological mutants of *A. tumefaciens* were isolated at a high frequency. This finding suggests that there are many factors affecting on morphogenesis in this organism. We are now investigating the biological and biochemical bases for the abnormal morphology of the mutants.

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