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Unidirectional Growth and Branch Formation of a Morphological Mutant, *Agrobacterium tumefaciens*

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Morphological characteristics of thermoconditional mutant *Agrobacterium tumefaciens* F-502 were investigated in relation to growth, division, and synthesis of cellular components. As a result of a shift from 27 to 37 C, mutant cells altered their morphology from short rods to elongated and branched forms; in addition, division and deoxyribonucleic acid synthesis were inhibited at 37 C. At 37 C unidirectional cell growth and branch formation occurred at one end of a cell, and the elongation rate of a cell was proportional to cell length. A hypothetical model for branch formation is presented in which the maximal elongation rate, 1.8 μm/h, at one end of a cell is an essential factor for initiation of branch formation.

Bacterial cell surfaces are composed of multiple layers, each having a definite arrangement. This structure determines cell morphology, such as shape and size. Recently, bacterial mutants that are filamentous or have an abnormal shape under restrictive conditions were isolated, and the genetic loci of determinative factors for cell morphology were mapped (15, 16, 18, 27, 32). Biochemical investigations of septum formation or cell elongation in relation to deoxyribonucleic acid (DNA) synthesis have been done (17, 25). Chemically, peptidoglycan, which is one component of the bacterial cell surface, is a determinative factor of cell form (20, 21, 39); however, no one can explain how it controls cell morphology. Henning et al. (14) obtained, from *Escherichia coli* K-12, rod-shaped "ghosts" which were free of murein; they hypothesized that certain envelope proteins are primarily responsible for the rod shape.

As another approach to morphogenesis, the division cycle was studied to obtain information about the growing site(s) or zone(s) of a cell. By immunofluorescence methods, it was determined that *Streptococcus pyogenes* and *Bacillus cereus*, gram-positive bacteria, grow symmetrically at one or a few sites or zones in a cell (4), and that *Salmonella typhosa* and *Escherichia coli*, gram-negative bacteria, also grow symmetrically at many sites by diffusive insertion of new cell surface materials (2). When autoradiographic methods were used, it was noted that cell walls of both *E. coli* and *B. megaterium* were synthesized diffusively (26, 28). Occurrence of random intercalation of new and old peptidoglycan in *B. subtilis* cell walls was also noted (29). However, Ryter et al. (34) reported the symmetric growth of *E. coli* K-12 in a central zone around a newly divided cell. Electron micrographs of *B. subtilis* and *B. megaterium* in thin sections suggested that cell wall synthesis occurs at a number of sites uniformly distributed along the cylindrical part of a cell but not at the polar regions (8). In a study on the distribution of phospholipid from parental to progeny cell membranes, it was reported that neither end (polar portions) of an *E. coli* cell is different from cylindrical portions (10). A similar result was obtained for *E. coli* by the density labeling method (38). On the other hand, the presence of a limited number of membrane-growing regions was indicated in temperature-sensitive *B. subtilis* flagella mutants (33). Adler and Hardigree (1) studied cell elongation with four strains of *E. coli* by the microculture method, a direct observation technique in which the progressive growth of individual cells is observed under a phase-contrast microscope. They discovered that three of the strains grew symmetrically at both ends and that one strain grew at one end only. Grula (12) also concluded from direct observation that the cell growth point of a strain of *Erwinia* sp. is the end formed by the previous division. Donachie and Begg (6) investigated the growth of *E. coli* T⁻JG151 by a microculture method and presented a "unit cell model" for bacterial growth. In this model, cells having a minimal length of about 1.7 μm are referred to as the "unit cell." The unit cell has a single envelope-growing site located at the pole (or end) formed during the

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previous cell division. Recently, by detecting cellular loci of the receptor site for phage T_6, they determined that the outer membrane of *E. coli* K12M6 grows only at two terminal zones (or sites) of a cell (3).

Previously (9), temperature-sensitive morphological mutants carrying the phenotype marker Bra$^+$ (positive branched-cell formation) were isolated from the wild-type F-010 of *Agrobacterium tumefaciens* IAM1525. The mutants grew normally, i.e., as short rods, at 27 C, whereas at 37 C they became elongated and branched. Branch formation means that a specific cell locus is morphologically marked with a branch (arm); therefore, mutants are considered useful for estimating growing sites (zones) and direction. In this paper, we describe the unidirectional growth and branch formation at one end of a mutant cell and present a novel conception of the biological significance of branch formation.

MATERIALS AND METHODS

Microorganisms, medium, and cultivation. *A. tumefaciens* F-010 and F-502 (9) were used as wild-type and morphology mutant strains, respectively. F-502, derived as a temperature-sensitive strain from F-010, shows a phenotype of Bra$^+$ (branched) at 37 C and Bra$^-$ (not branched) at 27 C. Yeast extract medium (Y medium) contained (per 100 ml): 1 g of yeast extract (Daigo-Eiyo Co., Osaka) and 5.0 ml of 1.0 M potassium-sodium-phosphate buffer, pH 7.0. Morphological change was traced with individual cells by the microculture method, as follows. A block (5 mm square, 0.5 mm thick) of Y medium containing 1% agar was placed on a sterilized glass slide, and the cells, precultured in liquid Y medium with shaking, were inoculated on the block. The cells were cultured at both 27 and 37 C. Changes in morphology during cultivation were observed at appropriate intervals with the aid of a phase-contrast microscope (Chiyoda Kogaku, Tokyo).

Determinations of DNA, RNA, total amino acids, and DAP. Bacterial cells harvested from liquid Y medium at the exponential growth phase (see Table 1) were washed with deionized water and used for determination of DNA, ribonucleic acid (RNA), total amino acids, and diaminopimelic acid (DAP). The amount of DNA and RNA, fractionated by the Schmidt-Trannhauser-Scheider method (35, 36), was determined by the indole method (19) and by measurement of absorbance at 260 nm (31), respectively. Total amino acids and DAP were determined in the cell hydrolysate as follows. Hydrolysis of cells was performed in a sealed tube containing 6 N HCl at 110 C for 20 h. After removal of HCl by repeated evaporation, the amount of each amino acid in the hydrolysate was determined with amino acid analyzer (automatic amino acid analyzer, no. AA-600; Shibata, Tokyo).

RESULTS

Morphological change of a temperature-sensitive mutant, F-502, by temperature shift-up. Morphology of strains F-502 (temperature-sensitive mutant) and F-010 (wild type) in microcultures was investigated at 27 and 37 C. Cells were inoculated from slants to liquid Y medium and precultured at 27 C with shaking. Those at an exponential growth phase (approximately 250 μg of cells/ml) were transferred onto an agar block of fresh Y medium and incubated at 27 or 37 C. To obtain the exact time course of various morphological events in the growth cycle, the progressive growth of individual cells was traced under a phase-contrast microscope. At 37 C, every cell of strain F-502 elongated to 5 to 6 μm in length, followed by branching at one end (Fig. 1). This finding suggests that the growing site (zone) resides at one end (or terminal) of a cell and that branch formation is a division of the growing site, because the two arms formed by branching grow at approximately equivalent rates. The cellular part (old arm), which had been already formed before branch formation, did not show any growth. Thus, there is clear evidence for occurrence of unidirectional or apical growth. At 27 C, the cells of strain F-502 had no internal marker indicating the intracellular locus; therefore, we could not obtain definite information about the direction and site of cell growth. During two division cycles, however, a cell of each strain grew into a microclone consisting of four cells arranged in parallel pairs on the agar medium (Fig. 2). We inferred from this arrangement that growth occurs unidirectionally at the new polar region (or end) formed by a previous division (see Discussion).

Relationship between growth rate and branch formation. When cell growth of individual cells was expressed as an increase in cell length and plotted on a semilogarithmic graph, linear lines with identical slopes were obtained as seen in Fig. 3 in which the length of each branched cell is the total length of the old and two new arms. The growth rate would thus be proportional to cell length and could be expressed as $dL/dt = K \cdot L$, where L is the cell length and K is the growth rate constant. The constant was temperature dependent in the microculture on the Y medium agar block, and the values were calculated to be 0.33 and 0.26 at 37 and 27 C, respectively, for strains F-502 and F-010 within 8 h of incubation. Figure 3 also indicates that branching occurred at a definite range of cell length, i.e., from 5.2 to 5.8 μm (average of 5.5 μm). The elongation rate at a

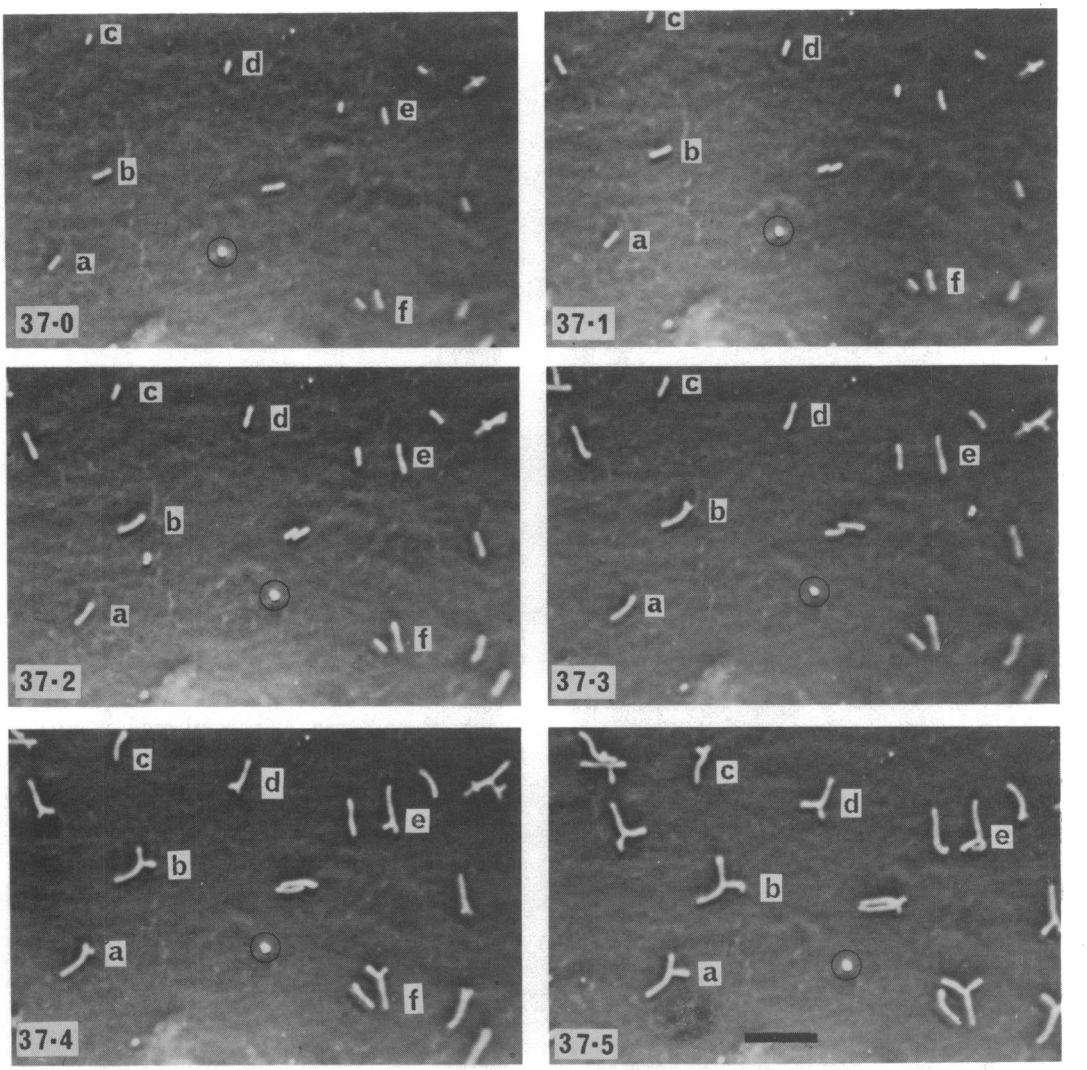

FIG. 1. *Progressive change in morphology of individual cells of strain F-502 in microculture at 37 C. Micrographs 37-0, 37-1, 37-2, 37-3, 37-4, and 37-5 indicate 0-, 1-, 2-, 3-, 4-, and 5-h cultures, respectively. Circle indicates an external marker. Bar, 10 μm.*

growing site, therefore, might be an initiation factor for branch formation.

Synthesis of cell materials during morphological change caused by temperature shift-up. To determine the biochemical basis for the morphological changes, we examined the effects of culture temperature on cell component synthesis. Before and after the cultivation at 37 C in liquid Y medium, the amount of DNA, RNA, and DAP and total amino acids in cells were determined (Table 1). There was a remarkable decrease in DNA content in mutant cells after cultivation for 4 h at 37 C. Synthesis of DNA, RNA, and protein was measured during cultivation and expressed as incorporation rates of labeled thymine, uracil, and leucine, respectively (T. Fujiwara, and S. Fukui, unpublished data); these data are in good agreement with those from Table 1.

Revertants from strain F-502 (leucine requiring, streptomycin resistant, and plant tumor inducing) grew in larger colonies than those of strain F-502 after 5 days of cultivation at 37 C (agar plates of Y medium), because the growth rate of strain F-502 decreased gradually as cultivation proceeded. The revertants, isolated as spontaneously reverted mutants, showed complete recovery of DNA-synthesizing ability

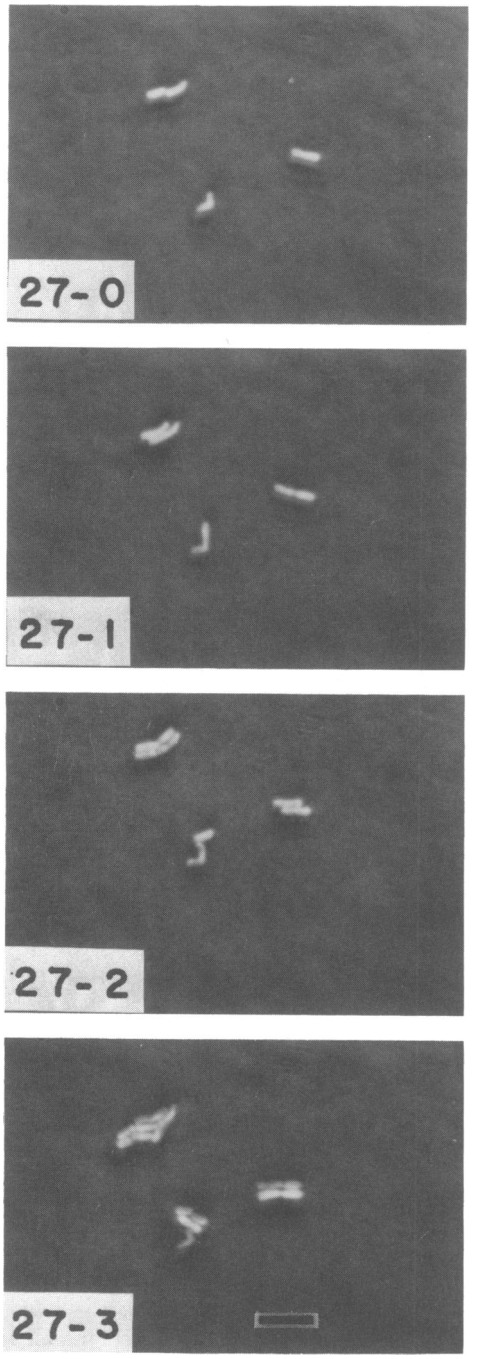

FIG. 2. *Cell arrangement during the formation of four-cell microclone from a single cell of F-502 in microculture at 27 C. Micrographs 27-0, 27-1, 27-2, and 27-3 indicate 0-, 1-, 2-, and 3-h cells, respectively. Bar, 5 μm.*

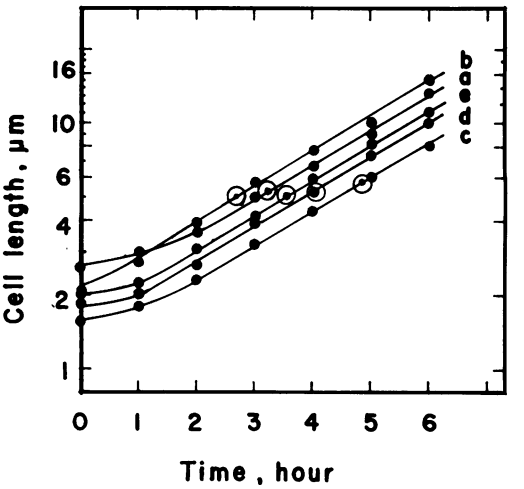

FIG. 3. *Elongation rate of individual cells in Fig. 1. Cell length was determined with enlarged micrographs (magnification, ×2,500). Open circle (○) indicates an apparent initiation point of branch formation. Curves a, b, c, d, and e show the time course of cell elongation of individual cells a, b, c, d, and e, respectively, which were named in Fig. 1.*

(Table 1) and grew as normal rods at growth rates equivalent to those of strain F-010 both at 27 and 37 C. The frequency of the reverse mutation was approximately 10^{-5}; thus, it is suggested that strain F-502 is a temperature-sensitive point mutant.

DISCUSSION

To determine the growing site (or zone) and growing direction in cells of *A. tumefaciens* strain F-502 during cultivation at 37 C in Y medium, we measured the distance between the branched sites relative to the ends of a cell (the internal marker method). We were able to demonstrate the occurrence of division inhibition, branch formation at one end of a cell, and unidirectional growth. At 27 C, however, branch formation did not occur, so that the internal marker method could not be applied. Thus, we extrapolated the growth model (unidirectional growth at one end of a cell) at 37 C to that at 27 C to construct the single-zone growth model described below.

When friction between the cell surfaces and agar and the interaction between cells are not considered, the arrangement of a four-cell microclone, which is derived from a single cell after two division cycles, can be expressed as a growth model. The arrangements expected from

typical growth models, fundamentally classified as symmetric or asymmetric, are schematically summarized in Fig. 4. When symmetric cell growth occurs at terminal zones of a cell, arrangement A, which gives "slipped parallel pairs" for a four-cell microclone, should appear. In asymmetric cell growth, especially in unidirectional growth at one end of a cell, four arrangements, B, C, D, and E, are possible. For process B, a cell grows unidirectionally and divides into two daughter cells which show unidirectional growth at the new end formed by division. In arrangement C, the growing end of the daughter cell is opposite the newly formed end. Arrangement D indicates that growing and nongrowing ends of a cell are permanently active and inactive, respectively. Arrangement E indicates that growing and nongrowing ends of a cell alternate in being the active and inactive ends at every division. In arrangements A, D, and E, a cell gives rise to a four-cell microclone with the same arrangement, i.e., designated "slipped parallel pairs." The morphology of four-cell microclones in arrangements B and C is designated "parallel pairs" and "series pairs," respectively.

Although more experiments are required to establish the significance of the four-cell microclone method for determination of growth models, we decided to analyze the growth model of *A. tumefaciens* by this technique. Formation of parallel pairs was always observed with strain F-502 at 27 C (Fig. 2) and with strain F-010 at both 27 and 37 C in the microcultures. Donachie

TABLE 1. *Change in cell components by a temperature shift from 27 to 37 C*[a]

Strain	Dry cell wt (μg/ml[b])	DNA (μg/ml[b])	RNA (μg/ml[b])	Total amino acids[c] (μg/ml[b])	DAP (absorbance at 570 nm)
Wild-type F-010					
Before	47	1.30	9.35	24.0	0.74
After	403	12.5	61.6	201	5.92
Mutant F-502					
Before	50	1.29	9.75	26.1	0.9
After	387	6.19	55.7	175	5.33
Revertant F-502-1					
Before	53	1.32			
After	420	10.7			

[a] Temperature shift-up was performed during the logarithmic growth phase (27 C) in Y medium, and cultivation was continued at 37 C. After 4 h of cultivation at 37 C, growth was at the middle logarithmic phase. Exponential growth was observed, during the 4-h cultivation period of both strains F-010 and F-502, by using a nephelometer (Kotaki, Co., Ltd., Tokyo) to measure turbidity.

[b] Per milliliter of culture medium.

[c] DAP was not included.

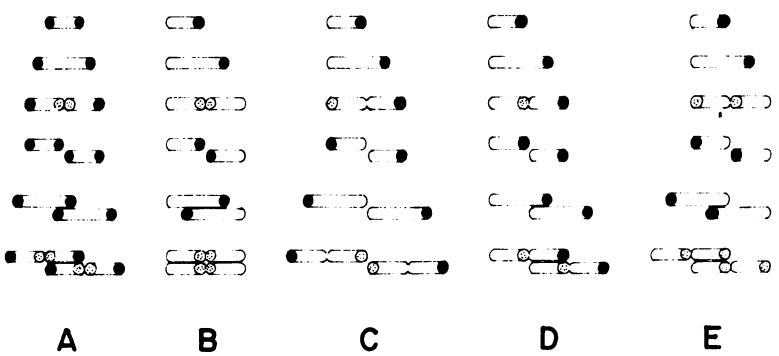

FIG. 4. *Presentation of polar zone growth model as cell arrangement during formation of four-cell microclone.* ◗ *and* ◗ *indicate growing zone and scheduled growing zone, respectively.*

and Begg (6) reported previously the formation of parallel pairs in *Escherichia coli* T⁻JG151 and conclusively stated that a cell grows unidirectionally in minimal medium at the end newly formed by division. Thus, we can explain the growth of strain F-502 at 27 C as follows.

A cell grows at one polar zone (or end) until its length reaches approximately 3 μm; it then divides into two daughter cells. Daughter cells thus formed slip out of alignment, and each cell continues to grow unidirectionally at the new polar zone formed by division. In this model (one polar zone growth model), cell growth may be directly associated with division, i.e., growth is initiated at a newly formed polar zone (or end) by completion of the previous division and is terminated by initiation of the next division. When the culture temperature was shifted from 27 to 37 C, inhibition of cell division was induced in the temperature-sensitive mutant F-502. According to the growth model presented above, the mutant cell can not terminate growth at 37 C and should continue to grow at a rate proportional to its length. The results presented in Fig. 3 agree with this prediction.

The characteristic change in cell morphology of strain F-502 was induced by temperature shift-up as follows. The cells elongated unidirectionally to approximately 5.5 μm without septum formation and then divided at the elongating end into two active ends. The initiation of the new end formation (branch formation), therefore, might be dependent upon the growth rate at the elongating end. An elongating rate of 1.8 ± 0.1 μm per h per growth zone was obtained in branch-initiating cells (Fig. 3 and unpublished data). We designated this as the "maximal rate." When the elongating rate surpassed the maximal rate, the cells could not maintain their characteristic morphology, rods. The bacterium then divided a growing site (zone), which was located at one end of a cell, into two active sites (zones) to slow down the elongation rate per active site. Thus, the growth rate expected from the equation given above could be maintained as the total rate of elongation in two new arms. Consequently, the bacterium changed cell form from rod to branched form. This model for branch formation is designated as "maximal growth rate model."

At 27 C, branched cells were never observed. The growth rate constant (K) of strain F-502 was 0.26 at this temperature; therefore, a cell length of 8 μm was required for the maximal rate (1.8 μm/h) to be reached. Cell division, however, took place at a cell length of 3.0 μm, at which the elongating rate was about 0.8 μm/h.

Contrasting data regarding the growth rate of a single cell were presented previously. *Bacillus cereus*, *Salmonella typhimurium*, and *Myxococcus xanthus* were shown to grow at rates proportional to their cell length (5, 7, 40) throughout one division cycle, whereas *E. coli* was shown to grow at constant rate (22, 23). We concluded that elongation rate of a single F-502 cell is consistent with the former view.

During the mophological alteration of strain F-502 at 37 C, a remarkable inhibition of DNA synthesis was observed (Table 1). However, direct correlation between cell morphology and DNA synthesis could not be made. An apparent relationship between DNA synthesis inhibition and formation of elongated cell (inhibition of septum formation) has been reported for *E. coli* and *B. subtilis* (11, 13, 30). The successive change in cell morphology observed here, such as elongation and branching, has not been reported previously, except for *E. coli* strain C in a medium containing mitomycin C (37). Morphological alteration in the cells of strain F-010 can be induced by addition of chemicals to the culture medium. Mitomycin C, a potent inhibitor of DNA synthesis, inhibits septum formation and induces branch formation at the growing pole of an elongated cell. D-Alanine, another effector of morphological change (24), alters short rods to tadpole-like forms. Details of these observations will be reported elsewhere.

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