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Novel Mutant Impaired in Cell Division: Evidence for a Positive Regulating Factor

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A novel, conditional, cell-division mutant from *Agmenellum quadruplicatum* strain BG1 is described. During rapid growth in dilute suspensions, cell division lags behind mass increase and the cells form filaments. These filaments spontaneously divide into unit cell lengths as the culture density increases. Other conditions that favor the accumulation of metabolic products in the medium antagonize filament formations. An 80% ethanol-water extract of dried, spent medium also restores the ability of filaments to divide into cells of unit length. Our results suggest that at least one chemical factor acting as a positive effector is involved in cell division.

Potentially, physiological investigations of bacterial mutants impaired in various aspects of the cell-division process could help to define and order the steps involved in the regulation of cellular division. This approach has been employed in the study of gram-negative organisms. A number of temperature-conditional mutants, which grow as serpentine filaments at the elevated temperature, have been described in *Escherichia coli* (4). Several explanations have been advanced for the failure of such mutants to divide at the nonpermissive temperature: impairment of deoxyribonucleic acid synthesis (2), alteration in membrane proteins (8), and destruction of a temperature-sensitive regulatory factor (12). Another class of mutants of *E. coli*, impaired very late in the cell division process, forms cross-septa but fails to separate into individual cells (10, 11).

Cell-division mutants of the blue-green bacteria have been much less intensively studied than have *E. coli* mutants. However, the blue-green bacteria may be unusually useful organisms for such studies. Morphologically aberrant mutants, presumably impaired in cell division, are recovered with high frequency after treatment of blue-green bacteria with nitrosoguanidine (5-7, 9). Filamentous mutants derived from the rod-shaped parent organisms show two distinct phenotypes: (i) septate filaments containing cross-walls, apparently impaired in the terminal stages of cell division, and (ii) serpentine forms which divide sporadically to produce coenocytic multinucleate filaments.

We are investigating cell-division mutants of the blue-green bacterium *Agmenellum quadruplicatum* strain BG1, which may be useful in defining the control mechanism of bacterial cell division. In this paper we describe a mutant that grows as multinucleoid filaments when agitated vigorously in dilute suspensions but returns to the wild phenotype (small rod-shaped cells) in dense suspensions. The ability of this mutant to divide normally can be restored in dilute suspensions by an extract of spent culture medium. This organism thus provides a unique system for the study of the positive regulation of a step in the division process.

MATERIALS AND METHODS

Organism and cultivation. *A. quadruplicatum* strain BG1 (a marine, unicellular, blue-green bacterium) was obtained as an axenic culture from C. Van Baalen (14). Strain 53S is a serpentine filamentous mutant of *A. quadruplicatum* strain BG1 isolated previously (7). The parent organism and its mutants were routinely grown autotrophically in ASP2 medium in culture tubes at 35 C or on petri plates solidified with 1% agar as previously described (7).

Mutagenesis. The SN12 mutant was obtained after treatment of the parent organism with nitrosoguanidine as described previously (7). It was initially selected for its ability to produce irregular colonies on agar medium.

Measurements of growth and cell number. Cell mass was routinely estimated by measurement of absorbance at 620 nm using a Spectronic 20 colorimeter with 22-mm culture tubes. Cell number was

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monitored by using a Coulter counter model B with a 30-μm aperture. Light micrographs were prepared by use of both phase and bright field microscopy.

Estimation of mean filament length. Stationary-phase cells from the parent were arbitrarily assigned a mean length of 1 (approximately 1 μmeter). Mean filament length was estimated by dividing the cell number per absorbance unit (620 nm) per unit volume of stationary-phase cells from the parent by that of the test population. By using this scheme, a population of parental cells in log phase has a mean filament length 2.4 times that of a stationary-phase population.

Preparation of division-promoting extract. Extracts were prepared from 2.4-liter batches of spent culture medium. Late log-phase cultures were harvested by centrifugation at 10,000 $\times$ *g* for 1 hr, and the cells were discarded. The supernatant fluid was concentrated by lyophilization in an industrial lyophilizer (Repp Industries, Gardiner, N.Y.). Dried medium was extracted with three portions (100 ml each) of 80% ethanol-water, and then concentrated by flash evaporation at 22 C. The syrupy residue was adjusted to a total volume of 15 ml by addition of distilled water.

RESULTS

Growth on plates. Cells of the parent organism form dense, round colonies with very regular margins (Fig. 1A). On agar, serpentine mutants typically form irregular colonies which are composed of very elongate multinucleoid cells (Fig. 1B). However, the irregular colonies of the SN12 mutant are composed of cells of normal morphology (Fig. 1C). Cells dividing normally would be expected to produce symmetrical colonies resembling the parent (Fig. 1A).

When appropriate dilutions of "normal cells" from macrocolonies of strain SN12 are spread on fresh plates and examined frequently during colony formation, the explanation for these irregular colonies is obvious (Fig 1D). Initially, the "normal cells" of SN12 grow into filaments which divide with increasing frequency as the colony develops. Thus, the initial filament formation produces the irregular margins which are subsequently preserved as the division process becomes more normal in the SN12 mutant. This suggests that substances which restore normal division ability may be accumulating during colony development.

Growth in liquid culture. In liquid culture, the filamentous mutant SN12 passes through a transitory filamentous condition analogous to that observed during colony formation on plates. After inoculation of culture tubes with "normal cells" from macrocolonies of strain SN12, or dilution of dense suspensions into fresh medium and incubation under growth conditions, cell division lags behind increases in cell mass, resulting in the production of filamentous cells (Fig. 3). Microscopic examination of these filaments reveals a very heterogeneous population exhibiting all morphological stages of the division process (Fig. 2A). As shown in Fig. 4, this filament formation is quite transient. As the culture increases in density, the filaments rapidly decrease in length. A much smaller, analogous variation in cell length occurs when stationary-phase cells of the wild-type organism are inoculated into fresh medium and incubated under growth conditions (Fig. 4). Both the parent and the SN12 mutant grow at the same rate at 35 C. In late-log cultures (48 to 60 hr, Fig. 4), the cells of the SN12 mutant approach the dimensions of wild-type cells (Fig. 2B and 2C). Cultures of SN12 can be maintained in the filamentous condition by frequent dilution into fresh medium. Similarly, cultures can be maintained as cells of normal dimensions by serial transfers of dense suspensions using large inocula.

Filament formation of the SN12 mutant in dilute suspension is strictly dependent upon vigorous agitation. Continuous agitation by rapid gassing is required for optimal filament formation (Fig. 5). Optimal filament formation can also be obtained by rapid agitation with a magnetic stirrer. The accentuation of filament formation in the SN12 mutant by treatments which presumably reduce any localized accumulation of diffusible extracellular material and the recovery of the cell's ability to divide normally as cultures increase in density, both in liquid and on agar, suggest the possible involvement of an externally accumulating factor as a limiting agent for the division of the SN12 mutant.

Activity of extract. The addition of an 80% ethanol extract of spent culture medium (from the parent or from the SN12 mutant) at the time of inoculation allows cell division in the SN12 mutant to proceed at a rate comparable to that of mass increase, antagonizing filament appearance (Fig. 6). Similar extracts prepared from (unused) autoclaved medium contained no detectable activity. At intermediate concentrations of the ethanol extract (20 to 30 μliters/ml), mean filament length is substantially reduced with little effect on increase in mass. Such treated cells average less than twice the mean length of logarithmically dividing cells from the parent organism (Fig. 4). Higher concentrations of this crude extract, though more effective at preventing filament formation, cause significant growth inhibition. As little as 20 μliters of extract per ml of growth medium partially restores normal cell division

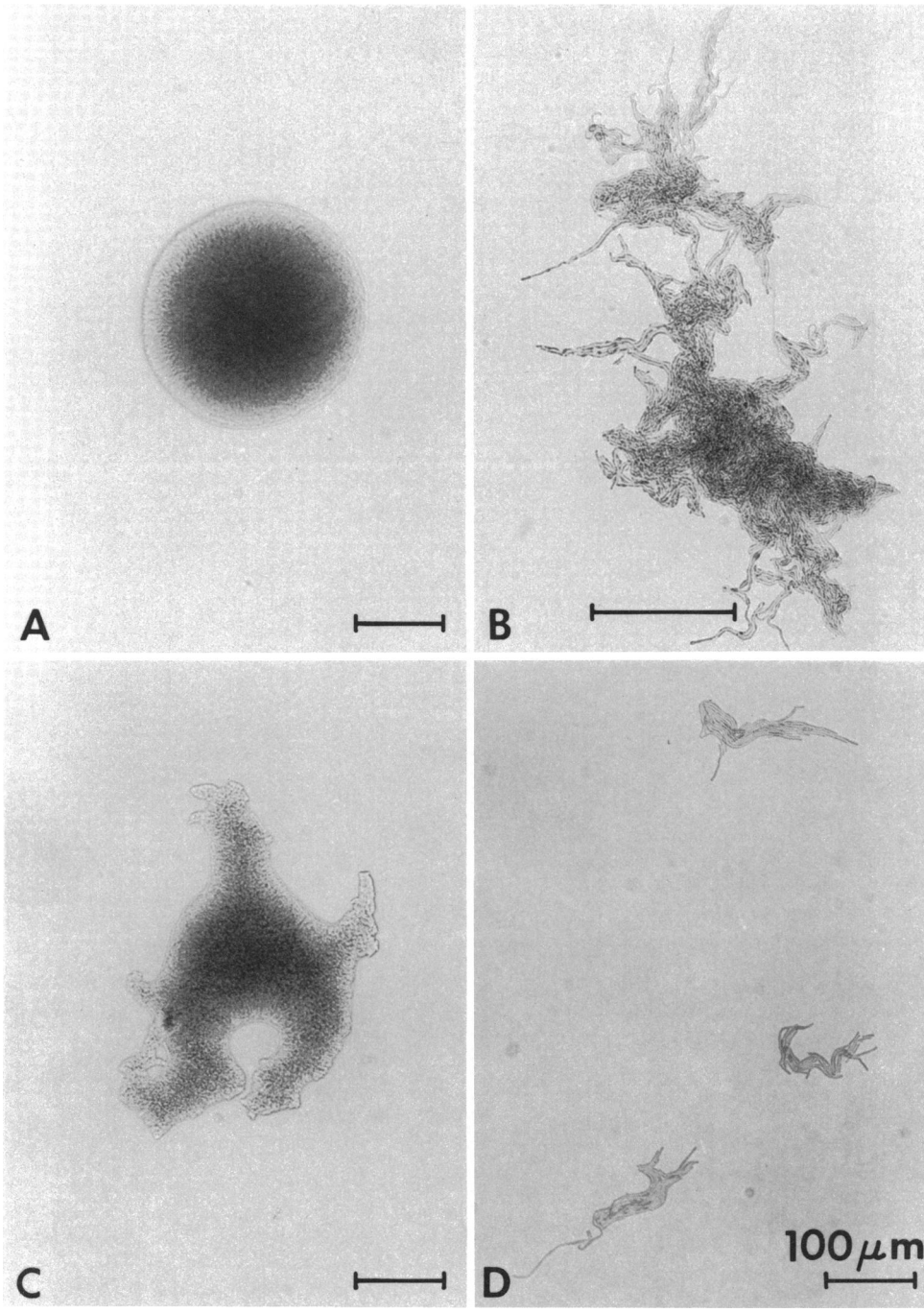

FIG. 1. *Growth of parent and mutants on agar plates. A, Wild-type macrocolony after 4 days; B, 53S macrocolony after 4 days; C, SN12 macrocolony after 4 days; D, SN12 mutant macrocolonies after 24 hr. Bar represents 100 μm.*

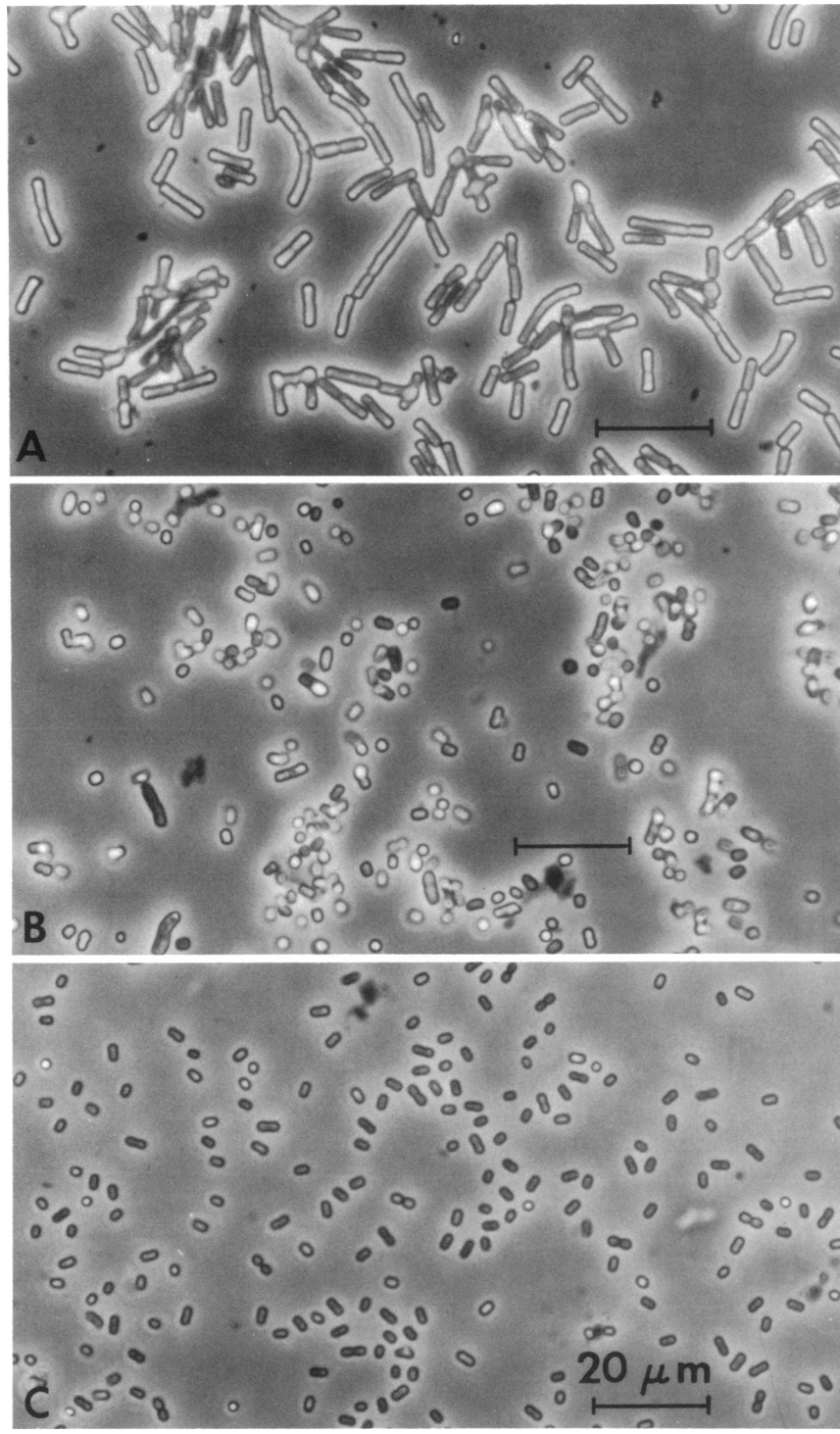

FIG. 2. *Phase-contrast micrographs of the SN12 filaments. A, After 24 hr of incubation in liquid; B, SN12 stationary-phase cells; C, the wild-type organism. Bar represents 20 μm.*

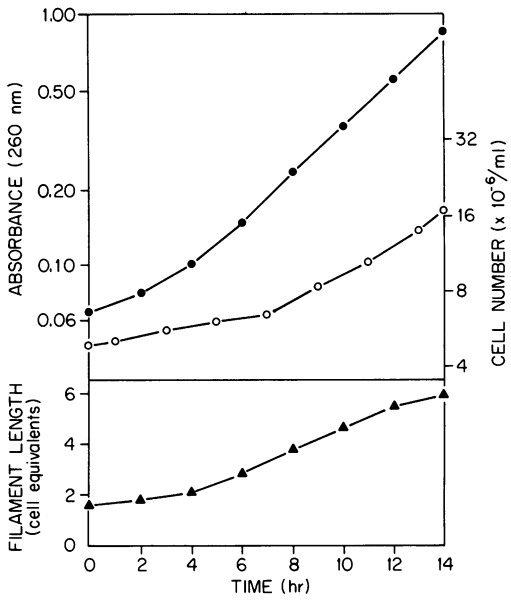

FIG. 3. *Effect of dilution on cell growth and filamentation. A 0.1-ml inoculum was diluted into 20 ml of fresh medium and incubated under growth conditions. The increase in cell mass (absorbance 0.06–0.85), ●, proceeds much more rapidly than the increase in cell number (4.8 → 1.7 × 10⁶/ml), ○, resulting in the production of filaments of increasing length (mean filament length 1.6 → 6.0), ▲.*

ability to the SN12 mutant, resulting in shorter filaments. Twenty microliters of extract corresponds to 3.2 ml of spent medium. Assuming a quantitative recovery of the active material, this represents only a 3.2-fold concentration from spent medium.

Growth of the SN12 mutant for 12 hr (log phase) in spent medium from a 48-hr culture (enriched with 10% fresh medium) resulted in the production of filaments only slightly shorter than those produced in fresh medium (12 hr). This observation suggests that the accumulation of cell-division-promoting material may not of itself be responsible for the spontaneous phenotypic recovery of the SN12 mutant during late-log and stationary-phase growth. This phenotypic recovery may be augmented by the general decrease in the rate of growth, allowing the impaired machinery of the division process to proceed and to restore the normal cellular dimensions.

DISCUSSION

Our results suggest that the SN12 mutant is partially impaired in the synthesis of some component which limits the rate of cell division without affecting the rate of mass increase. Biologically active material accumulates in spent medium from both the parent and the SN12 mutant, presumably by diffusion out of dividing cells. This active material is prepared by extracting lyophilized medium with 80% ethanol. Fresh medium contains no active material. Preparations from 24-hr cultures are less active than those from 48-hr cultures. The addition of the extract from spent medium to cultures of strain SN12 restores their division ability. Conditions that potentially enhance the diffusion of waste products or metabolites out of the cell (i.e., rapid agitation or dilution) accentuate the cell-division impairment of the SN12 mutant.

As liquid cultures or colonies on agar plates become increasingly crowded, the ability of the SN12 mutant to divide normally is restored. This synergistic action of cells is similar to the phenomenon termed "neighbor restoration" (3). After irradiation of *E. coli* B, populations

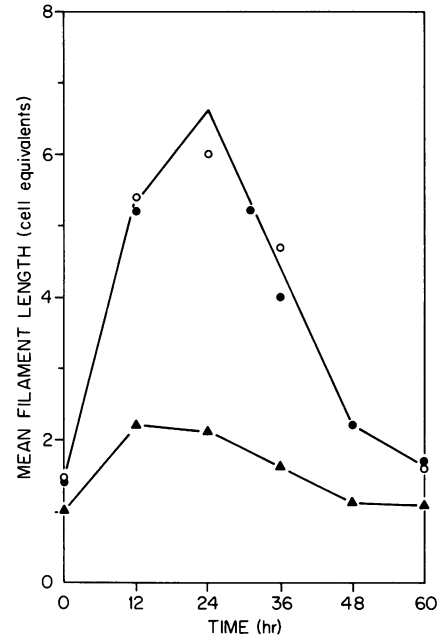

FIG. 4. *A comparison of the developmental cycle of the SN12 mutant to that of the wild-type organism in liquid culture. Samples (0.03 ml) from stationary-phase cultures of SN12, ● and ○ (separate experiments), and from the parent organism, ▲, were diluted into fresh medium and monitored during subsequent incubation under growth conditions. Mean filament length was estimated as equivalent cell lengths by a comparison of the ratios of cell number per absorbance unit. Stationary-phase parent cells were assigned the value of 1.*

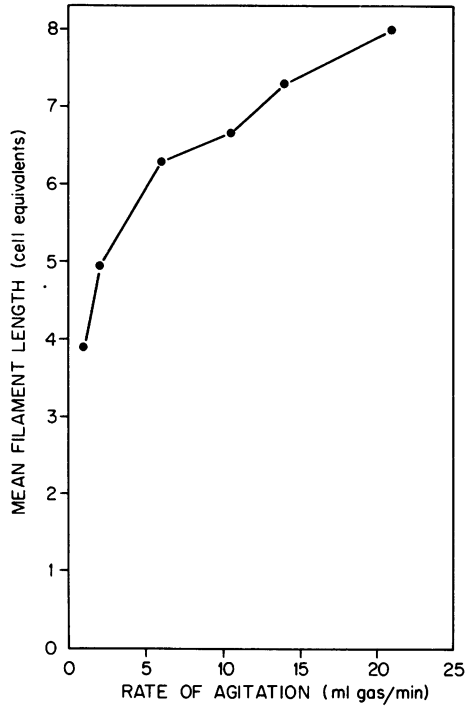

FIG. 5. *Effect of the rate of agitation on the filament formation of the SN12 mutant. A stationary-phase culture was diluted 1:1,000 into fresh medium. Samples were incubated at various levels of aeration for 24 hr.*

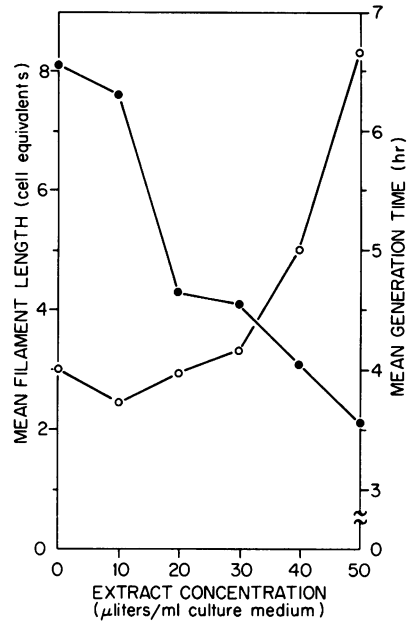

FIG. 6. *Effect of ethanol extract on growth and cell division of the SN12 mutant. A stationary-phase suspension of SN12 was diluted 1,000-fold into fresh medium. Samples were incubated for 24 hr at 35 C in the presence of various concentrations of the ethanol extract. The effects of this extract on growth are presented as mean generation times (○). Mean filament length (●) was determined after incubation.*

of filament-forming cells impaired in cell division partially recover division ability if tightly grouped on agar surfaces (1). An active fraction has been isolated from whole cells of *E. coli* that also enhances cell division and survival of irradiated cells (1). It has been suggested that the phenomenon of "neighbor restoration" results from the action of diffusible substances leaking from groups of cells that stimulate cytokinesis (1). Our results with the SN12 mutant demonstrate that a diffusible substance produced by both the parent and the mutant accumulates in the medium and restores the regulation of cell division. This suggests that cell division may be regulated at some level by a positive effector that is subsequently excreted. An analogous system with a positive effector (cyclic adenosine monophosphate) may exist in the regulation of inducible enzymes by *E. coli* (13).

We realize that more quantitative data concerning the accumulation of the proposed positive effector would be desirable. However, the complications of recovering quantitatively the active material from an artificial sea-water medium coupled with the difficulty in handling the SN12 mutant prevent such an analysis at this time. We are currently attempting to isolate a more manageable cell-division mutant. By using such a mutant, we hope to isolate the cell-division-promoting component from the ethanol extract for further chemical and biological characterization.

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