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A Pleiotropic Mutation Affecting Expression of Polar Development Events in *Caulobacter crescentus*

(mutant selection/antibody to flagellin/surface structures/coordinate assembly)

NURITH KURN, SUSAN AMMER, AND LUCILLE SHAPIRO

Department of Molecular Biology, Division of Biological Sciences, Albert Einstein College of Medicine, Bronx, New York 10461

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ABSTRACT **A developmental mutant of *C. crescentus* with altered polar surface structures has been isolated. The mutant lacks a flagellum and pili, and may have an abnormal DNA phage receptor site. A revertant regains the normal structures simultaneously. This point mutation allows normal flagellin synthesis, stalk formation, equatorial cell division, and rate of growth. The mutant phenotype indicates that the assembly of the polar surface structures is coordinately regulated and independent of mechanisms regulating cell polarity and division.**

The unicellular prokaryote, *Caulobacter crescentus*, characterized by a slender stalk at one pole of the cell, undergoes a series of defined structural changes during each division cycle (1, 2). Just prior to division, the cell elaborates a flagellum (3), pili (2, 4), and a DNA phage receptor site (2, 5). All of these alterations occur at the cell pole opposite the stalk, so that equatorial division yields dissimilar daughter cells. A motile *swarmer cell* is derived from the flagellated pole and a sessile *stalked cell* from the original stalked pole. A subsequent morphological alteration occurs when the *swarmer cell* sheds its flagellum (3) while synthesizing a new stalk at the same site (6), thereby effecting the morphogenesis of a *swarmer* to *stalked cell* (see Fig. 5). We have concentrated our efforts on elucidating the first of these major morphogenic events, the site-specific synthesis of various polar organelles just prior to cell division. The simultaneous expression of elucidating, pili, and DNA phage receptor site (2–5) at a defined pole of the cell suggests that the appearance of these organelles may be coordinately controlled. Additional evidence was obtained by physiologically interfering with the division cycle. The normal pattern of morphogenesis is altered upon changing the carbon source in minimal medium from glucose to lactose or galactose. Development in the culture is coordinately arrested at the nonmotile *elongated stalked cell stage*, just prior to the expression of the polar functions (8). The inhibition of development caused by the change of carbon source from glucose to lactose or galactose can be overcome spontaneously (after 20–30 hr) or by addition of dibutyryl cyclic AMP or glucose to the minimal growth medium (8).

We report here the isolation of a pleiotropic mutant exhibiting coordinate alteration of the surface polar structures. The isolation of this mutant provides additional evidence for the coordinate control of surface morphogenesis.

MATERIALS AND METHODS

Growth Conditions. Caulobacter crescentus strain CB13B1a was grown at 30° in either minimal medium (8) containing one of a variety of carbon sources (0.2%, w/v), or nutrient broth PYE (8). Bacteriophage ϕCbK and ϕCb5 were cultured as previously described (5, 9).

Isolation of Mutants. Wild-type bacteria were grown to mid-logarithmic phase in PYE at 30°. After addition of 100 μg/ml of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (10) the cultures were grown for 15–30 min at 30°. The viable count on PYE agar plates was reduced by about 80%. Single colonies were transferred to fresh nutrient broth and checked for motility with the Zeiss phase contrast microscope. Twelve non-motile mutants were isolated. Two of these non-motile mutants, SM-4 and SM-5, were found to be resistant to infection by the RNA phage ϕCb5 and showed abnormal infection by DNA phage ϕCbK (cloudy plaques on PYE agar plates). The mutant phenotype was maintained during growth in nutrient broth, as well as in minimal medium with glucose, galactose, xylose, ribose, mannose, lactose, maltose, or sucrose as a carbon source. In all cases the growth rates of wild-type, mutant, and revertant cultures were essentially equivalent. Spontaneous streptomycin-resistant (str^r) strains of SM-4 and SM-5 were then isolated in order to provide an additional genetic marker. In the following we shall refer to only one of the strains, SM-4, carrying the str^r marker, since we were unable to demonstrate any significant differences between SM-4 str^r and SM-5 str^r, or between SM-4 and SM-4 str^r.

Isolation of Revertants. The isolation of revertants was facilitated by the use of a modification of the chemotaxis assay described by Adler (11). Single colonies of non-motile mutants grown on PYE agar plates were transferred to PYE broth and grown at 30° until $OD_{660} = 0.1$ (Zeiss spectrophotometer). Aliquots (0.1 ml) of this culture were transferred to serum test tubes. Sterile capillaries were filled with 1% glucose, sealed at one end and placed upright in the test tubes (11). After 1-hr incubation at room temperature the contents of each capillary, enriched in motile cells attracted to the glucose, were inoculated into 5 ml of sterile PYE broth and incubated overnight at 30°. This process was repeated six times. Cultures were then plated on PYE agar. Single colonies were transferred to PYE soft agar plates (0.35% agar). Motile revertants and wild-type cells formed large colonies as opposed to the small colonies formed by the non-motile mutants (Fig. 1). Motile revertants were then grown in PYE broth and checked for sensitivity to RNA and DNA phage infection (2, 5, 9) and maintenance of streptomycin resistance.

Preparation of Flagellin. C. cresentus CB13 or SM-4, grown to mid-logarithmic phase (0.5 OD units), were harvested by

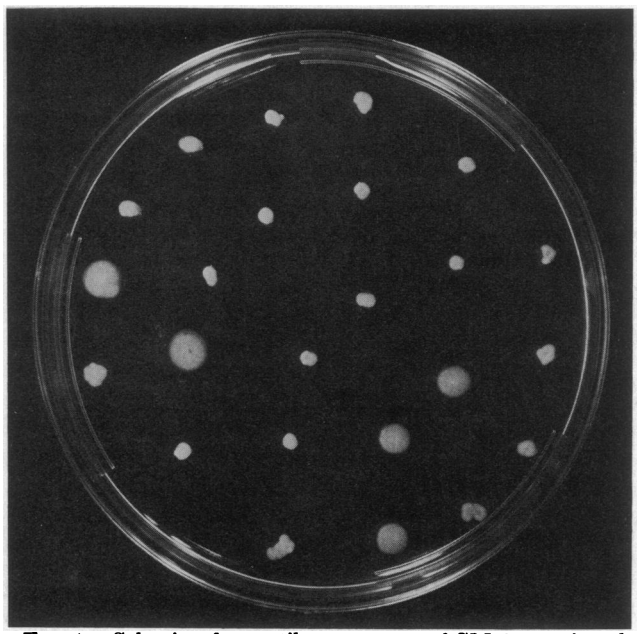

FIG. 1. Selection for motile revertants of SM-4 carrying the str^r marker. Single colonies from mutant cultures enriched with motile bacteria by chemotaxis toward glucose (see *text*) were transferred to PYE soft agar (0.35%) plates and grown overnight at 30°. Colonies of the motile revertant strain were marked by a large diameter as opposed to the small colonies of the mutant strain.

centrifugation at 10,000 × *g* for 10 min and frozen. Frozen cells (4 g) were ground with 8 g of alumina and the resulting paste was suspended in 15 ml of 0.15 M NaCl. After centrifugation for 10 min at 10,000 × *g* the supernatant fluid (8 mg of protein per ml) was either heated at 55° for 45 min and then centrifuged for 10 min at 10,000 × *g* to remove insoluble material, or was adjusted to pH 2.0 with 1 N HCl, mixed for 60 min at room temperature, and then centrifuged as above.

For preparation of anti-flagellin antiserum, intact flagella were isolated from the culture fluid of late-logarithmic phase cells as described previously (3). Purified flagellin was prepared by the procedure of McGroarty *et al.* (13), in which flagella are first solubilized at pH 2 and then allowed to reaggregate at pH 7.2. Samples for either immunoprecipitation or sodium dodecyl sulfate–polyacrylamide gel electrophoresis were heat dissociated (45 min at 55°) prior to analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the purified flagellin showed a single band corresponding to a polypeptide chain with a molecular weight of 25,000 (3).

Anti-flagellin serum was obtained from rabbits injected with purified flagellin mixed with an equal volume of Freund's complete adjuvant. The immunoglobulin was partially purified by heating to 65° for 10 min followed by precipitation with ammonium sulfate added to 50% of saturation and dialysis against saline.

TABLE 1. *Phenotype of development mutant SM-4*

	Wild-type CB13	Mutant SM-4 str^r	Revertant SM-4 str^r rev
Motility	+	−	+
Flagella*	Present	Absent	Present
RNA phage φCb5 infection titer (PFU)§	1×10^5	N.D.†	7.7×10^5
Pili*	Present	Absent	Present
DNA phage φCbK‡ infection titer (PFU)§	2.4×10^{10}	2.1×10^9	3.6×10^{10}
plaque morphology	Clear	Cloudy	Clear
Generation time (min) in PYE	80	80	83

* By electron microscopy.

† Not detected.

‡ φCbK lysate prepared by infection of wild-type host.

§ A known quantity of phage (as measured on wild-type host) was plated on mutant and revertant host strains at the same multiplicity of infection, under standard assay conditions (5, 9).

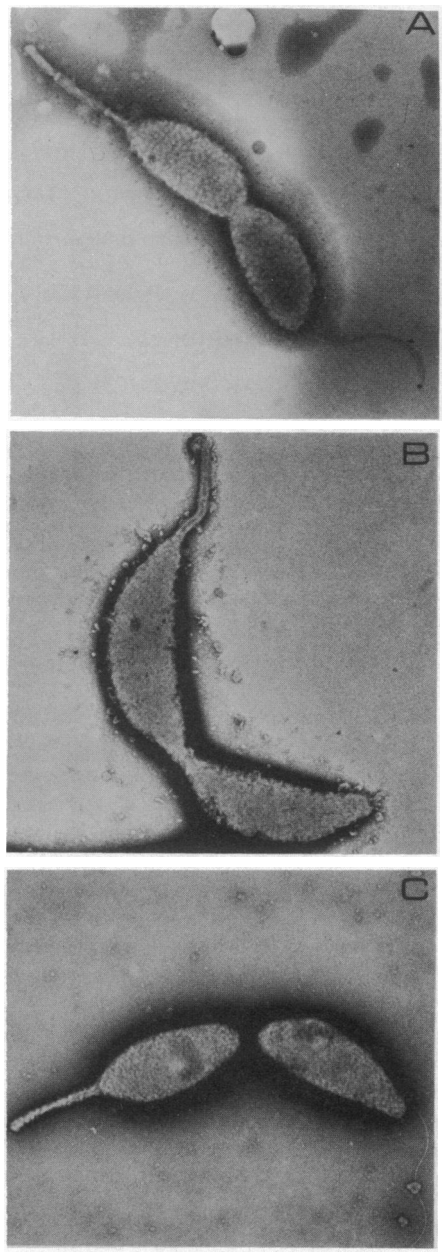

FIG. 2. Electron micrographs of *C. crescentus* CB13 (*A*), SM-4 mutant (*B*), and its revertant (*C*). Cells grown in nutrient broth (PYE) were stained as described in *Materials and Methods*.

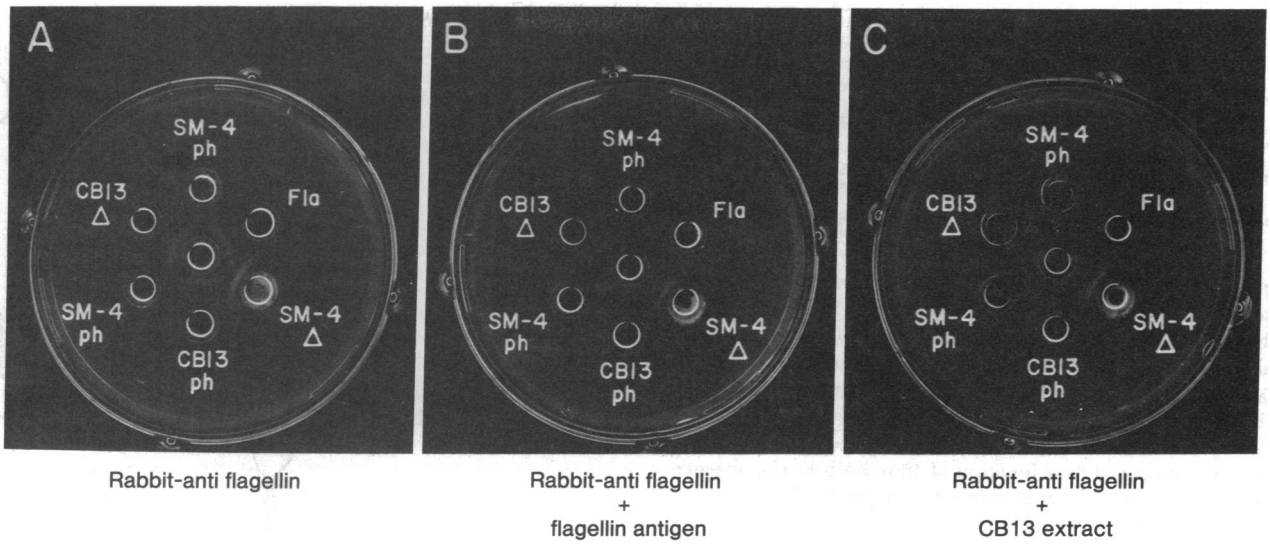

FIG. 3. Gel diffusion analysis of cell-free extracts of *C. crescentus* CB13 and SM-4. Immunodiffusion plates were prepared using 60-mm plastic plates containing 5.0 ml of 0.8% agarose. Wells of 25-μl volume were cut prior to use. The rabbit antisera raised against purified *C. crescentus* CB13 flagellin were concentrated 2-fold by $(NH_4)_2SO_4$ precipitation. In the outer wells of plate *A*, as indicated, the antigens were 4 μg of flagellin (*Fla*), 71 μg of *C. crescentus* SM-4 heated (Δ) cell extract, 40 μg of *C. crescentus* CB13 acid- (*ph*) and heat-treated cell extract, 47 μg of SM-4 acid- and heat-treated cell extract, 68 μg of CB13 heat-treated cell extract, and 43 μg of SM-4 acid and heat-treated cell extract, and the center well contained rabbit anti-flagellin antiserum. The antigen wells in plates *B* and *C* were the same as those in plate *A*, but the center well in plate *B* contained antiserum plus purified flagellin and that in plate *C* contained antiserum plus CB13 acid- and heat-treated cell extract.

Electron Microscopy. Bacterial preparations were stained with 0.5% sodium silicotungstate at pH 7.0 (12) on a 200 mesh carbon-coated copper grid, as described previously (3). A Siemens Elmskop 1A electron microscope at a voltage of 80 kV was used for this study.

RESULTS

Phenotype of Development Mutants. A group of non-motile mutants of *C. crescentus* have been isolated which vary in the production of flagella precursor, flagellin, as well as in the coincident appearance of polar surface structures. One of these mutants, M-2, lacked flagellin but possessed pili, appeared normal, and was an efficient host for the RNA phage φCb5 and the DNA phage φCbK (3). Other mutants synthesized flagellin but failed to assemble the flagellum; these mutants were normal with respect to other polar structures and phage infection. In contrast, the non-motile mutants SM-4 and SM-5 exhibited altered phage receptor function. This was manifested by the production of cloudy plaques upon DNA bacteriophage φCbK infection and the lack of RNA bacteriophage φCb5 infection (Table 1). Efficiency of plating of φCbK on SM-4 host was found to be an order of magnitude lower than on a wild-type culture. The precise chemical nature of the DNA phage φCbK receptor site remains an open question, although mutant analysis indicates that intact flagella or pili are not required for adsorption or infection. The aberrant infection of SM-4 with DNA bacteriophage φCbK could be due to an alteration in the phage receptor site, yielding a reduced affinity for phage adsorption. This would result in a lowering of the number of successful infections by progeny phage and in turn yield cloudy plaques. The optical density (660 nm) of infected mutant cultures did not decrease, in contrast to the lysis exhibited by infected wild-type cultures. Resistance to infection by the RNA bacteriophage φCb5 can be correlated with the absence of pili, since pili are the normal RNA phage receptor sites. The lack of SM-4 motility correlated with the absence of assembled flagella (Fig. 2), but, as will be shown, flagellin was synthesized. Motile revertants of SM-4 and SM-5 strains were isolated which regained the wild-type phenotype with regard not only to presence of pili and flagella, but also to sensitivity to RNA phage φCb5 infection and normal infection by DNA phage φCbK (Table 1 and Fig. 2).

Flagellin Synthesis. Electron micrographs revealed the total absence of assembled flagella in mutant cultures (Fig. 2). Cell extracts were analyzed by both sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunodiffusion in order to determine if the precursor protein, flagellin, was synthesized by the SM-4 mutant.

The isolation and gel electrophoretic pattern of flagellin has been previously described (3). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of total proteins from cells of both mutant and wild-type cultures showed the presence of flagellin. No significant change in total proteins between the mutant and wild-type could be detected by this method. The presence of flagellin in cell extracts of mutant cultures was also demonstrated by immunodiffusion analysis using rabbit anti-flagellin antisera (Fig. 3*A*). Antigens derived from intact wild-type flagella, as well as total soluble flagellin from acidified cell extracts of wild-type and mutant cultures, exhibited identical precipitin patterns. An additional precipitin band, corresponding to antigen of a higher molecular weight than that of flagellin monomers, was observed when soluble flagellin was prepared from heated, but not from acidified, mutant cell extracts. This additional precipitin band was not observed with similarly prepared wild-type cell extracts. A mixture of anti-flagellin antisera and either purified flagellin or acid-soluble cell extract of the wild-type strain caused the disappearance of all precipitin bands tested except the band corresponding to the extra antigen in heat-treated mutant cell extracts

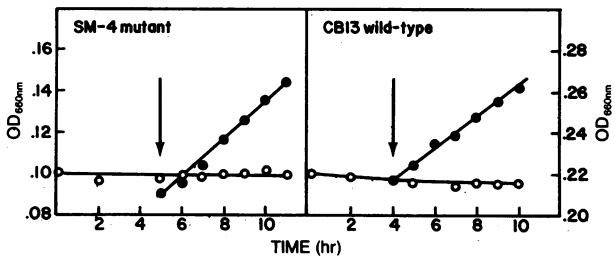

FIG. 4. The effect of carbon source shift from glucose to lactose on the growth of *C. crescentus* CB13 and SM-4 and rescue by dibutyryl cyclic AMP. *C. crescentus* SM-4 or CB13 was grown to mid-logarithmic phase in minimal medium plus glucose (0.2%); cells were collected by centrifugation at 10,000 × *g* for 10 min, and the pellet was gently resuspended in prewarmed minimal medium containing 0.5% lactose. The optical density at 660 nm was measured as a function of time both in the absence (○) and presence (●) of 3 mM dibutyryl cyclic AMP.

(Fig. 3*B* and *C*). The nature of this antigen is unclear at present.

The presence of flagellin protein in mutant cell extracts suggests that the defect in SM-4 is in assembly of the flagellum and not the actual expression of the flagellin structural gene. It is still necessary to define the synthesis and assembly of pili in order to demonstrate alteration in the assembly of other surface structures.

Effect of Dibutyryl-3',5'-Cyclic AMP. It had previously been observed that upon a switch from glucose to lactose, minimal medium wild-type cultures block both growth and development at the elongated stalked cell stage just prior to the appearance of the surface polar structures (8). Since growth and normal development resumed upon the addition of exogenous dibutyryl cyclic AMP (8), it was of interest to repeat these experiments with the polar development mutant SM-4. After transfer of a mid-logarithmic culture of SM-4 from glucose to lactose minimal media, there was a small drop in optical density at 660 nm, which stabilized after 4–6 hr (Fig. 4). The mutant culture came to rest at the elongated stalked cell stage. This block in cell growth and cell division, similar to that of a wild-type (8) and revertant culture, was overcome spontaneously after 20–30 hr or by the addition of dibutyryl cyclic AMP (3 mM). The addition of dibutyryl cyclic AMP, however, did not restore assembly of flagellum, pili, and normal RNA and DNA phage infection in the mutant culture. Cell growth induced by exogenous dibutyryl cyclic AMP can, therefore, occur independent of the assembly of polar structures.

DISCUSSION

In the work presented here we described the phenotype of a mutant altered in the pattern of development during the *C. crescentus* cell cycle (see Fig. 5). Although isolated on the basis of motility alone, this mutant was found to be defective in several polar functions, i.e., the absence of flagellum occurs concomitantly with resistance to RNA phage φCb5 infection, lack of pili, and aberrant infection with DNA phage φCbK. Revertant cultures were found to regain all polar functions simultaneously.

We suggest that the basic defect in this mutant is in the assembly mechanism related to transfer or localization of the gene products. As opposed to other non-motile, flagella-less

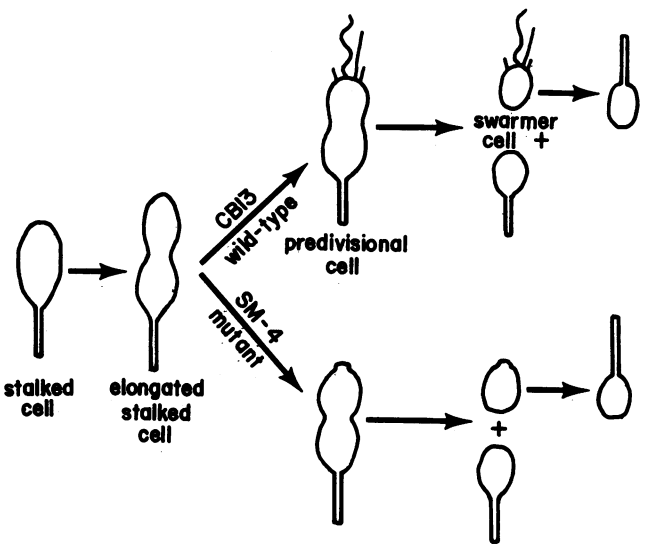

FIG. 5. Schematic representation of the cell cycle of *C. crescentus* wild-type CB13 and mutant SM-4.

mutants isolated, SM-4 was found to synthesize the precursor molecule, flagellin. Precursor molecules for the phage infection sites have not, as yet, been identified. The aberrant assembly of the surface polar structures could result from an alteration of a site-specific, membrane-bound, regulatory mechanism while maintaining the overall structural and functional integrity of the cell membrane.

The coordinate expression of these polar functions can be physiologically blocked, i.e., upon transfer of a mid-log phase culture from glucose to lactose minimal medium (8). Cells at all stages of the cell cycle during mid-logarithmic phase growth in glucose simply continue around the cell cycle and accumulate at the elongated stalked cell stage upon shift from glucose to a carbon source which requires the induction of catabolic enzymes. The blocked culture maintains viability but stops growth and cell division, and appears unable to express the surface differentiation events, including flagella, pili, and phage receptor site formation at the cell pole. This growth and development block is overcome spontaneously, or by addition of dibutyryl cyclic AMP. As with wild-type cultures, mutant cultures blocked at the elongated stalked cell stage upon a switch from lactose to glucose, and the addition of dibutyryl cyclic AMP to the mutant culture permitted normal growth and cell division. In the mutant cultures, however, rescue occurred in the absence of the assembly of the surface polar structures, demonstrating that the cell cycle is independent of surface structure formation.

It was previously demonstrated that the synthesis of flagellin, a protein subunit of the assembled flagella, coincides with the appearance of the polar surface structures (3), indicating a correlation between gene activity, determination of polar site, and assembly of the surface structure. The existence of a coordinate mutant altered in surface structures while preserving normal polarity, cell division, and rate of growth, indicates that these structures are not obligatory to the *C. crescentus* cell cycle. Moreover, since stalk synthesis occurred at the proper site on the cell and at the correct time in the cell cycle in this mutant, polarity is independent of the actual assembly of the other polar surface structures.

In summary, the phenotype of the developmental mutant SM-4 indicates that the polar structures, flagellum, pili, and DNA phage receptor site, are coordinately controlled and that cell polarity and division are independent of the actual assembly of these structures.

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