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Caulobacter crescentus Pili: Structure and Stage-Specific Expression

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Pili are functionally expressed during the predivisional and swarmer stages of the *Caulobacter crescentus* differentiation cycle. They appear on the developing swarmer pole and at the same cellular location as flagella and the φCbK receptor sites. Pili disappear when the swarmer cell differentiates into a stalked cell; this occurs with the loss of flagella and the disappearance of phage receptor sites. *C. crescentus* CB13B1a pili have been purified and characterized. Monomeric pilin is a protein with an apparent molecular weight of 8,500 that stains weakly with periodic acid-Schiff reagent. The amino acid composition of purified pilin reveals very low quantities of basic amino acids and a complete absence of methionine. Pilin is synthesized throughout the *C. crescentus* differentiation cycle. Neither free pili nor pilin monomers are detectable in the growth media, suggesting that loss of piliation in the swarmer- to stalked-cell transition occurs via pilus retraction.

Pili are thin surface filaments that have been identified in a variety of unrelated bacterial genera (10, 16, 18, 21, 35, 37), although physical characterization of these structures is limited to only a few species (3, 10, 11, 18). The function of pili in many of these species is unknown. Some pili appear to be involved in bacterial conjugation (10, 11, 25); others may be involved in pathogenicity (34), whereas still others may facilitate bacterial survival under adverse conditions (10). Pili from diverse bacterial genera have further been shown to serve as receptors for ribonucleic acid (RNA) phage (5, 13, 27) and deoxyribonucleic acid phage (8, 21, 22).

Caulobacter pili were first identified by J. Schmidt in species able to adsorb *Caulobacter*-specific RNA phage (27). Pili were observed almost exclusively at the flagellated pole of swarmer cells, although pili were not observed on every swarmer cell (27). Further evidence of the stage-specific expression of piliation is derived from an analysis of the adsorption of RNA phage φCb5 to synchronized *Caulobacter crescentus* (CB13B1a) populations (29). These studies have shown that RNA phage adsorb specifically to predivisional- and swarmer-stage cells and not to stalked cells. Electron microscopy has further confirmed the stage specificity of piliation (unpublished data).

In this report we identify and characterize *C. crescentus* pilin, the structural protein of pili. We further demonstrate that the 8,500-molecular-weight pilin monomer is synthesized throughout the *Caulobacter* differentiation cycle. Aspects of the stage-specific expression of pili in relation to this finding are discussed.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *C. crescentus* CB13B1a and a derivative, SW16-Pil 200, were used. SW16 was a spontaneous mutant, originally selected as a nonmotile colony able to grow on a confluent lysis plate of φCbK. Upon cloning, this strain proved to be sensitive to φCbK and supersensitive to the RNA phage φCb5. Supersensitivity to φCb5 was assessed by a fivefold increase in plating efficiency and increased clarity of plaques. SW16 is further characterized by a lack of both flagellin subunits (20) and is defective in stalk formation. Cells were grown in modified (1) peptone-yeast extract (26) for pili purification or in glucose minimal media (31) for pulse-labeling studies. Liquid cultures were incubated at 30°C on a rotary shaker.

Pili purification. Pili were routinely purified from 8 liters of mid-exponential-phase cultures of SW16 (absorbance at 600 nm, 0.5 to 0.7). Cultures were rapidly cooled over ice and centrifuged at 16,000 × *g* for 10 min. All subsequent steps were carried out at 4°C. Cell pellets were suspended in 800 ml of 10 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 8.2; 200-ml portions were sheared for 45 s at top speed in a Waring blender. Cell bodies were removed by centrifugation at 16,000 × *g* for 10 min. Pili were concentrated and purified from this cell-free supernatant by 30 to 45% saturated $(NH_4)_2SO_4$ precipitation. The $(NH_4)_2SO_4$ concentration was adjusted by dropwise addition of saturated $(NH_4)_2SO_4$ to the stirring cell-free supernatant. The precipitates were harvested by centrifugation at 23,000 × *g* for 15 min, resuspended in 10 mM Tris-hydrochloride, pH 8.2, and dialyzed overnight

against 2 liters of the same buffer. Material insoluble after dialysis was removed by centrifugation at 16,000 × g for 10 min. The supernatant fluid (containing pili) was then adjusted to 0.1 M $MgCl_2$ and stored overnight at 4°C. The 0.1 M $MgCl_2$ precipitate was harvested by centrifugation at 23,000 × g for 15 min and suspended in 1 ml of 10 mM Tris-hydrochloride, pH 8.2. Pilus preparations were adjusted to 34% (wt/vol) CsCl (Harshaw Chemical Co.) and centrifuged at 75,000 × g for 50 h in a Beckman SW50.1 rotor at 5°C. Pili formed a visible band at ρ = 1.236 g/cm³. Pili prepared from CB13B1a exhibited a protein profile identical to that of SW16 pili, but yields of pili were poorer, and flagella were persistent contaminants in preparations from wild-type cells.

Electrophoresis. Pilus purity was routinely assayed by sodium dodecyl sulfate (SDS)-polyacrylamide discontinuous gel electrophoresis (PAGE) by the general procedure of Laemmli (19) modified as previously described (36). High resolution of low-molecular-weight proteins was obtained using an SDS-urea-PAGE slab gel system (Castillo et al., J. Mol. Biol., in press). These gels contained a 12 to 15% gradient of acrylamide (acrylamide-bis, 40:2) and a 12 to 42% gradient of urea. SDS-urea-PAGE gradients were poured in slabs 20 cm long and polymerized at 4°C. Gels were stained with Coomassie brilliant blue as described previously (20) or with periodic acid-Schiff reagent by the Segrest and Jackson method (28).

Amino acid analysis. Purified pili (see Fig. 2 and 3D and E) were hydrolyzed in 6 N HCl at 110°C for 24 h. Analysis was carried out with a Durrum D 500 amino acid analyzer with the technique of Spackman et al.(33).

RESULTS

Identification of pilin protein. Electron microscopy was used to monitor purification of intact pili since the structural protein of pili (pilin) had not been identified. It was thus determined that pili could be precipitated in the presence of 50% saturated ammonium sulfate. When this crude preparation was subjected to CsCl equilibrium density gradient centrifugation, a visible band composed of pili (Fig. 1) was observed at ρ = 1.236 g/cm³. Protein profiles of these preliminary steps in the purification displayed by 15% SDS-PAGE in the Laemmli (19) system revealed enrichment of a protein with an apparent molecular weight of 8,500. Subsequently, 0.1 M $MgCl_2$ (12) was found to selectively precipitate this protein and, likewise, precipitate intact pili as determined by electron microscopy. When $MgCl_2$ precipitation was used prior to centrifugation in CsCl, homogeneous pilin could be prepared (Fig. 2). Details of the routine pilus purification procedure based on these findings are described above. When shearing of the cells was omitted, pili could not be recovered in supernatant fractions, suggesting that these structures are not normally released into the culture medium at the end of the swarmer stage. Furthermore, cell-free culture media, concentrated by lyophilization, contain large amounts of released flagellin (20) but no detectable pilin.

Physical characterization of pili. The buoyant density of pili is 1.236 g/cm³, suggesting the possibility that pili might contain carbohydrate. Periodic acid-Schiff staining of cylindrical gels produced a faint positive reaction in the pilus band region. The nature and extent of this apparent carbohydrate modification are under investigation. SDS-urea-PAGE (Castillo et al., J. Mol. Biol., in press) was employed to obtain better resolution of proteins in the low-molecular-weight range of *C. crescentus* pilin. The SDS-urea-PAGE profile of the final steps in pilin purification, as described above, are shown in Fig. 3; with this technique, crude pilus preparations appear to be enriched for proteins with apparent molecular weights of 7,500 and 8,500. CsCl equilibrium density gradient centrifugation further enriches for the 8,500-molecular-weight protein in the pilus band at ρ = 1.236 g/cm³. The relationship between these proteins is currently under investigation.

The amino acid composition of CsCl-purified pili composed of the 8,500-molecular-weight polypeptide is shown in Table 1. *Caulobacter* pili completely lack methionine and have very low levels of basic amino acids. Glycine and alanine account for one-third of the total amino acid residues.

Synthesis of pilin in synchronous populations. *C. crescentus* CB13B1a contains relatively few proteins in the molecular-weight range of pilin; therefore, this protein can be meaningfully assayed in whole-cell extracts by the SDS-urea-PAGE system. To determine the time of synthesis of pilin during the CB13B1a differentiation cycle, cells grown in glucose minimal medium were synchronized and pulse-labeled with ^{14}C-amino acids at 10-min intervals. Labeled whole-cell proteins were displayed by SDS-urea-PAGE. The patterns of labeled proteins, revealed by autoradiography (Fig. 4), indicate that the 8,500-molecular-weight pilin is synthesized throughout the CB13B1a differentiation cycle. The 7,500-molecular-weight protein, which copurifies with the pili prior to CsCl centrifugation, appears to be preferentially labeled during the swarmer stage (0 to 60 min); labeling of this 7,500-molecular-weight protein is significantly reduced during the stalked (60 to 120 min) and predivisional (120 to 180 min) stages.

The labeling pattern of pilin is distinct from that of another stage-specific, polar, structural protein, flagellin. The 25,000-molecular-weight

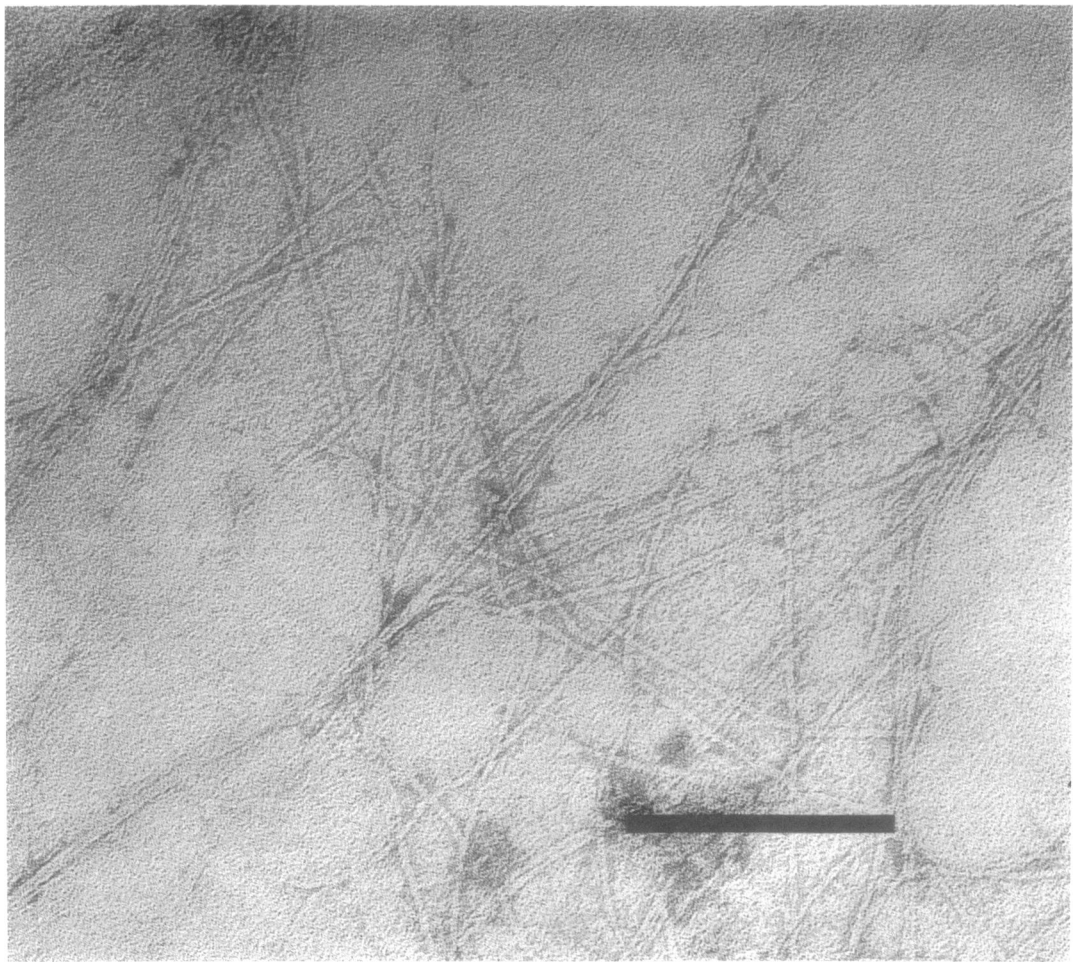

FIG. 1. *Intact pili isolated by CsCl equilibrium density gradient centrifugation and stained with 2% uranyl acetate, pH 4.2, as previously described (36). Bar indicates 0.2 μm.*

flagellin is synthesized only during the predivisional and early swarmer stages and is rapidly polymerized after synthesis (32). The high resolution obtained with SDS-urea-PAGE reveals numerous proteins that exhibit stage-specific synthesis. Especially apparent in the autoradiogram shown in Fig. 4 are those proteins below a molecular weight of 13,000 whose synthesis is limited to distinct periods during the first 40 min of the swarmer-cell stage.

DISCUSSION

Caulobacter pili are flexible, filamentous protein structures found uniquely at the swarmer-cell pole. *C. crescentus* pili are similar to those of *Pseudomonas aeruginosa* PAO1, not only in their polar location, but also in their dimensions and appearance in the electron microscope (6): *C. crescentus* pili are about 4.0 nm in diameter (27) and 1 to 4 μm in length; they do not appear to be hollow, and they increase in number after cells are infected with noncontractile-tailed DNA phage that adsorb at the piliated-cell pole (20a). *C. crescentus* pilin (molecular weight, 8,500) is the smallest pilus monomer characterized to date. Its amino acid composition reveals an absence of methionine, very low quantities of basic amino acids, and large amounts of glycine and alanine. A similar absence of methionine has been demonstrated in type I pili of *Escherichia coli* K-12 (10). The amino acid composition of both type I and F pili of *E. coli* is also low in basic amino acids and high in nonpolar amino acids (10, 11). Type I pili of *E. coli* (10) and pili of *Neisseria gonorrhoeae* (12), *Corynebacterium renale* (18),

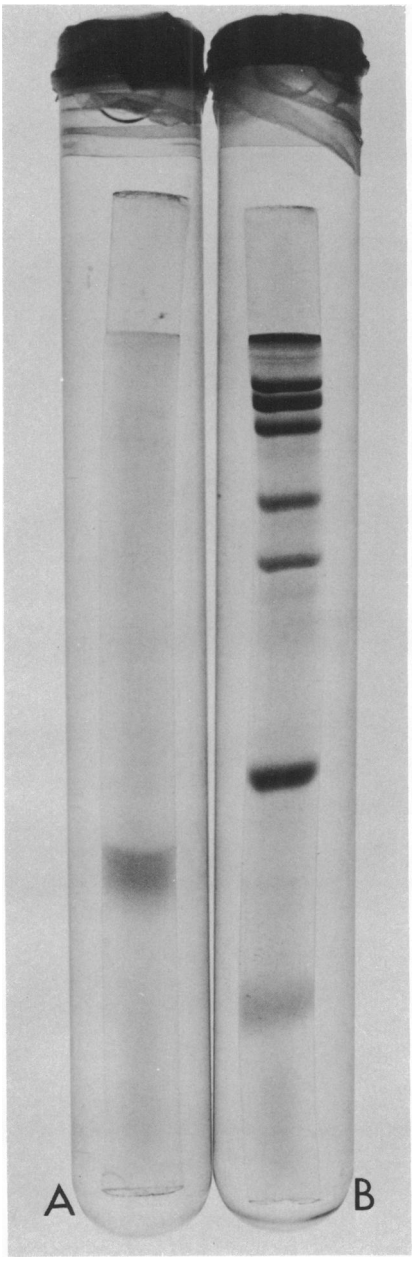

FIG. 2. *SDS-PAGE of purified pili. (A) Pili purified through final CsCl equilibrium density gradient step; (B) molecular weight standards from top to bottom are 100,000, 80,000, 66,500, 41,000, 31,000, 13,600, and 6,000, respectively.*

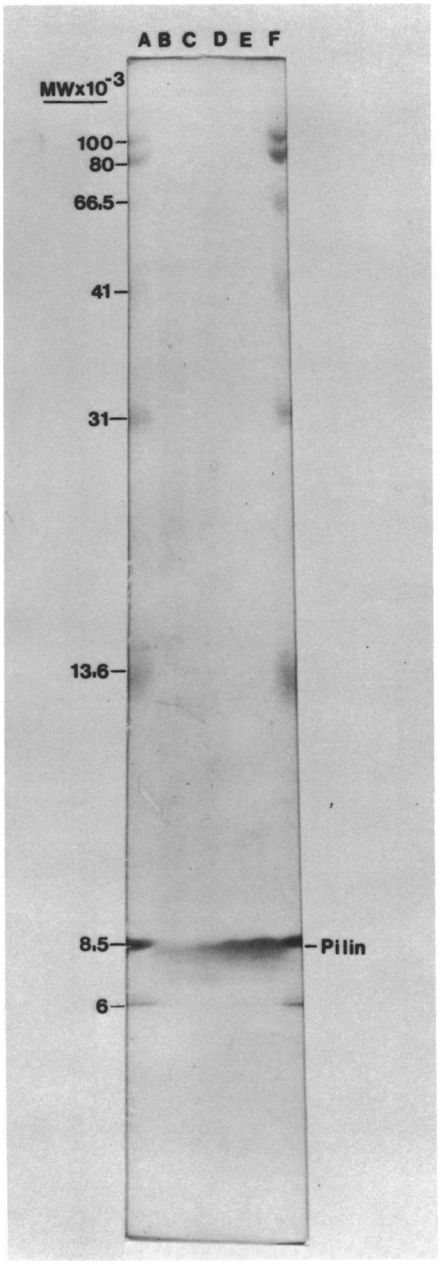

FIG. 3. *SDS-urea-PAGE of final steps in pilus purification (see Materials and Methods). (A) and (F) Molecular weight standards; (B) and (C) $MgCl_2$-precipitated pili; (D) and (E) CsCl equilibrium density gradient-purified pili.*

and *C. crescentus* are precipitable by 0.1 M $MgCl_2$.

The buoyant density of *Caulobacter* pili is 1.236 g/cm^3, and purified preparations are stained by periodic acid-Schiff reagent, indicating the presence of carbohydrate in these preparations. This compares favorably with the buoyant density of F pili of 1.257 g/cm^3: F pili

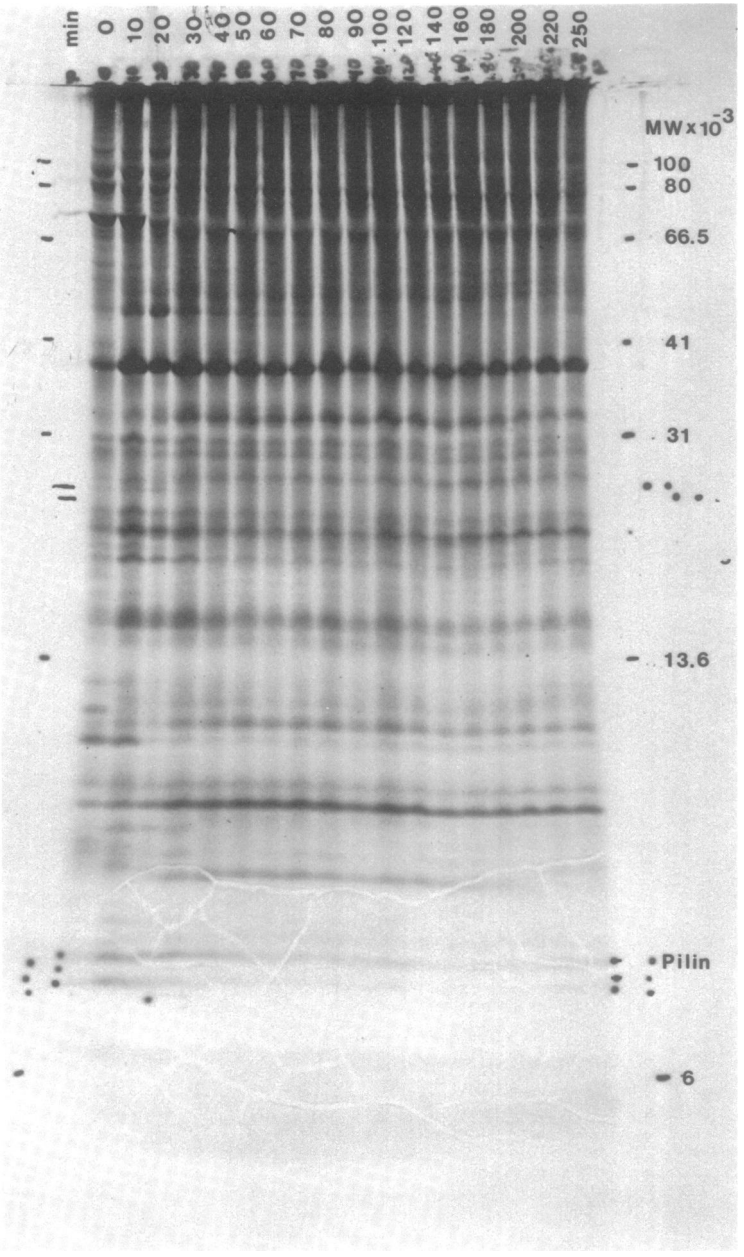

FIG. 4. *Pilin synthesis in CB13B1a: SDS-urea-polyacrylamide gel autoradiogram of pulse-labeled C. crescentus. Synchronous CB13B1a swarmer cells were obtained by the Ludox technique (15). Two-milliliter portions (absorbance at 660 nm, 0.1) were pulsed for 5 min with 5 μCi of ^{14}C-labeled amino acids (reconstituted protein hydrolysate; New England Nuclear) and chased for 2 min with 2 ml of 10% Casamino Acids. Labeled cells were pelleted at 12,000 × g for 10 min and prepared for electrophoresis as previously described (20).*

contain a single glucose residue and two phosphate residues per pilin monomer (11). Autoradiography of whole-cell extracts labeled with ^{32}P and fractionated by SDS-urea-PAGE fails to show any indication of phosphorylation of *C. crescentus* pilin (unpublished data).

Studies of *E. coli* (24) and *P. aeruginosa* (7, 9) suggest that pili are dynamic structures that

TABLE 1. *Amino acid composition of pilin*

Residues	Amt (residues/protein)
Asp	4
Thr	10
Ser	6
Glu	7
Pro	2
Gly	12
Ala	13
Val	5
Met	0
Ile	3
Leu	6
Tyr	1
Phe	2
His	1
Lys	2
Arg	1

may be extended and retracted in a process that, in part, appears to be energy dependent (24). Direct evidence of extension and retraction is not available because pili cannot be microscopically resolved in living bacteria. Furthermore, the systems in which pili have been characterized do not exhibit cell cycle-dependent regulation of piliation. *Caulobacter* provides a unique system for studying pilus biogenesis, since the external appearance and disappearance of these organelles are developmentally controlled (27, 30).

In the course of normal differentiation, the piliated swarmer cell develops into a nonpiliated stalked cell; our studies indicate that this does not occur by the shedding of pili into the culture fluid, as is the case with *Caulobacter* flagella (20, 32). To examine the level of regulation of pilus biogenesis in *Caulobacter*, synchronous populations of cells were pulse-labeled at 10-min intervals throughout the differentiation cycle. The results of these studies (Fig. 4) indicate that the pilin monomer is synthesized continuously by both stalked and swarmer cells; thus, the stage-specific appearance of pili is not achieved by regulating the synthesis of pilin. Since stalked cells synthesize pilin, but do not possess pili, pilin must either be stored in cellular reservoirs or rapidly and specifically degraded by the stalked cell. Beard and Connolly (2) have identified pilin pools in the isolated outer membrane of *E. coli*; similar studies in this laboratory have demonstrated that a minor protein with a molecular weight corresponding to pilin may be found in purified outer membranes from heterogeneous populations of CB13B1a.

The function of pili in *Caulobacter* remains a topic of conjecture. At least two interesting possibilities exist that may be investigated as a result of the identification and characterization of the pilin structural protein: pili may function to define a sexually differentiated cell form in the *Caulobacter* life cycle, and/or it may facilitate bacterial survival under adverse conditions, as proposed for the function of type I pili in *E. coli* (10).

Cell-mediated gene transfer has been demonstrated in several *Caulobacter* strains; however, no direct evidence exists for the involvement of pili in the gene transfer process. In all reported cases of *Caulobacter* gene transfer (17, 23; N. Agabian-Keshishian, Ph.D. thesis, Albert Einstein College of Medicine, New York, 1971, and C. Ruby, MS thesis, Indiana University, Bloomington, 1967), one of the parental species is *C. crescentus* strain CB15, a piliated strain (27). We have shown that the CB15 pili are similar to CB13B1a pili in molecular weight and are likewise synthesized throughout the cell cycle. CB15 pili are also receptors for the RNA bacteriophage ϕCb5, which is able to infect both CB15 and CB13B1a strains (4, 27). Although some plasmid-coded pili of *E. coli* are involved in conjugation (11), *C. crescentus* plasmids have not yet been found.

The cell types that participate in conjugation in *Caulobacter* are also not known; however, the presence of pili on swarmer cells may designate these cells as participants and, by analogy, donors in the cell-mediated transfer of genetic material. This represents an interesting dilemma regarding the mechanism(s) of gene transfer in *Caulobacter* since deoxyribonucleic acid replication does not occur in the swarmer stage of the cell cycle (14).

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LITERATURE CITED

1. **Agabian-Keshishian, N., and L. Shapiro.** 1971. Bacterial differentiation and phage infection. Virology 44:46–53.
2. **Beard, J. P., and J. C. Connolly.** 1975. Detection of a protein, similar to the sex pilus subunit, in the outer membrane of *Escherichia coli* cells carrying a derepressed F-like R factor. J. Bacteriol. 122:59–65.
3. **Beard, J. P., T. G. B. Howe, and M. H. Richard.** 1972. Purification of sex pili from *Escherichia coli* carrying a derepressed F-like R factor. J. Bacteriol. 111:814–820.
4. **Bendis, I., and L. Shapiro.** 1970. Properties of *Caulo-*

bacter ribonucleic acid bacteriophage ϕCb5. J. Virol. 6:847–854.

5. **Bradley, D. E.** 1966. The structure and infective process of a *Pseudomonas aeruginosa* bacteriophage containing ribonucleic acid. J. Gen. Microbiol. 45:83–96.
6. **Bradley, D. E.** 1972. A study of pili on *Pseudomonas aeruginosa*. Genet. Res. 19:39–51.
7. **Bradley, D. E.** 1972. Shortening of *Pseudomonas aeruginosa* pili after RNA-phage adsorption. J. Gen. Microbiol. 72:303–319.
8. **Bradley, D. E.** 1973. A pilus-dependent *Pseudomonas aeruginosa* bacteriophage with a long non-contractile tail. Virology 51:489–492.
9. **Bradley, D. E.** 1974. The adsorption of *Pseudomonas aeruginosa* pilus-dependent bacteriophages to a host mutant with non-retractile pili. Virology 58:149–163.
10. **Brinton, C. C., Jr.** 1965. The structure, function, synthesis and genetic control of bacterial pili and a molecular model for DNA and RNA transport in gram negative bacteria. Trans. N. Y. Acad. Sci. 27:1003–1054.
11. **Brinton, C. C., Jr.** 1971. The properties of sex pili, the viral nature of "conjugal" genetic transfer systems, and some possible approaches to the control of bacterial drug resistance. Crit. Rev. Microbiol. 1:105–160.
12. **Buchanan, T. M., J. Swanson, K. K. Holmes, S. J. Kraus, and E. C. Gotschlich.** 1973. Quantitative determination of antibody to *Gonococcal* pili. J. Clin. Invest. 52:2896–2909.
13. **Crawford, E. M., and R. F. Gesteland.** 1964. The adsorption of bacteriophage R17. Virology 22:165–167.
14. **Degnen, S. T., and A. Newton.** 1972. Chromosome replication during development in *Caulobacter crescentus*. J. Mol. Biol. 64:671–680.
15. **Evinger, M., and N. Agabian.** 1976. Stage-specific transitions of the *Caulobacter crescentus* folded chromosome. Fed. Proc. Fed. Am. Soc. Exp. Biol. 35:1585.
16. **Fuerst, J. A., and A. C. Hayward.** 1969. Surface appendages similar to fimbriae (pili) on *Pseudomonas aeruginosa* species. J. Gen. Microbiol. 58:227–237.
17. **Jollick, J. D., and T. Q. Tran.** 1975. Polarity of gene transfer in *Caulobacter*. J. Gen. Microbiol. 91:183–187.
18. **Kumazawa, N., and R. Yanagawa.** 1972. Chemical properties of the pili of *Corynebacterium renale*. Infect. Immun. 5:27–30.
19. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680–685.
20. **Lagenaur, C., and N. Agabian.** 1976. Physical characterization of *Caulobacter crescentus* flagella. J. Bacteriol. 128:435–444.

20a. **Lagenaur, C., S. Farmer, and N. Agabian.** 1977. Adsorption properties of stage-specific *Caulobacter* phage ϕCbk. Virology 77:401–407.

21. **Lotz, W., and H. Pfister.** 1975. Attachment of a long-tailed *Rhizobium* bacteriophage to the pili of its host. J. Virol. 16:725–728.
22. **Marvin, D. A., and B. Hohn.** 1969. Filamentous bacterial viruses. Bacteriol. Rev. 33:172–209.
23. **Newton, A., and E. Allebach.** 1975. Gene transfer in *Caulobacter crescentus*: polarized inheritance of genetic markers. Genetics 80:1–11.
24. **Novotny, C. P., and P. Fives-Taylor.** 1974. Retraction of F pili. J. Bacteriol. 117:1306–1311.
25. **Ou, J. T., and T. F. Anderson.** 1970. Role of pili in bacterial conjugation. J. Bacteriol. 102:648–654.
26. **Poindexter, J. S.** 1964. Biological properties and classification of the *Caulobacter* group. Bacteriol. Rev. 28:231–295.
27. **Schmidt, J. M.** 1966. Observations on the adsorption of *Caulobacter* bacteriophages containing ribonucleic acid. J. Gen. Microbiol. 45:347–353.
28. **Segrest, J. P., and R. L. Jackson.** 1972. SDS gel electrophoresis of glycoproteins. Methods Enzymol. 28:54–63.
29. **Shapiro, L., and N. Agabian-Keshishian.** 1970. Specific assay for differentiation in the stalked bacterium *Caulobacter crescentus*. Proc. Natl. Acad. Sci. U.S.A. 67:200–203.
30. **Shapiro, L., N. Agabian-Keshishian, and I. Bendis.** 1971. Bacterial differentiation. Science 173:884–892.
31. **Shapiro, L., N. Agabian-Keshishian, A. Hirsch, and O. M. Rosen.** 1972. Effect of dibutyryladenosine 3'5'-cyclic monophosphate on growth and differentiation in *Cauolobacter crescentus*. Proc. Natl. Acad. Sci. U.S.A. 69:1225–1229.
32. **Shapiro, L., and J. V. Maizel, Jr.** 1973. Synthesis and structure of *Caulobacter crescentus* flagella. J. Bacteriol. 113:478–485.
33. **Spackman, D. H., W. H. Stein, and S. Moore.** 1958. Automatic recording apparatus for use in the chromatography of amino acids. Anal. Chem. 30:1190–1206.
34. **Swanson, J.** 1973. Studies on *Gonococcus* infection. IV. Pili: their role in attachment of *Gonococci* to tissue culture cells. J. Exp. Med. 137:571–589.
35. **Tweedy, J. M., R. W. A. Park, and W. Hodgkiss.** 1968. Evidence for the presence of fimbriae (pili) on *Vibrio* species. J. Gen. Microbiol. 51:235–244.
36. **West, D., C. Lagenaur, and N. Agabian.** 1976. Isolation and characterization of *Caulobacter crescentus* bacteriophage ϕCd1. J. Virol. 17:568–575.
37. **Wistreich, G. A., and R. F. Baker.** 1971. The presence of fimbriae (pili) in three species of *Neisseria*. J. Gen. Microbiol. 65:167–173.