AD-A184 152 LOCALIZING THE SUBUNIT POOL FOR THE TEMPORALLY REGULATED POLAR PILI OF CAULOBACTER CRESCENTUS(U) CALIFORNIA UNIV OAKLAND NAVAL BIOSCIENCES LAB J SMIT

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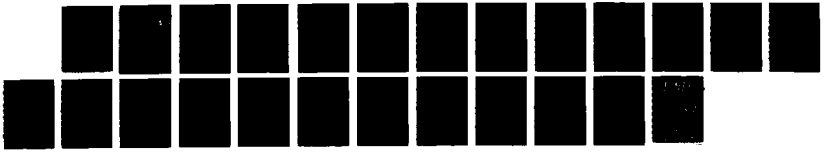
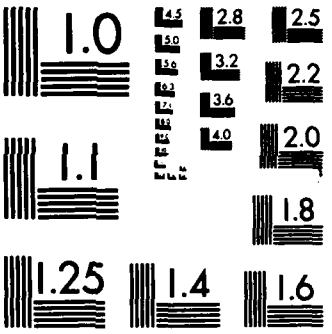
UNCLASSIFIED 1987 UC-NBL-952 N00014-81-C-0570 F/G 6/13 NL





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SECURITY CLASSIFICATION OF THIS PAGE

MENTATION PAGE

AD-A184 152

1a. REPORT SECURITY CLASSIFICATION: UNCLA	1b. RESTRICTIVE MARKINGS: NONE
2a. SECURITY CLASSIFICATION AUTHORITY: N/A	3. DISTRIBUTION/AVAILABILITY OF REPORT: Approved for public release; distribution unlimited
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE: N/A	
4. PERFORMING ORGANIZATION REPORT NUMBER(S): NBL No. 952	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION: University of California	6b. OFFICE SYMBOL (If applicable):
6c. ADDRESS (City, State, and ZIP Code): Naval Biosciences Laboratory, Naval Supply Center, Oakland, California 94625	7a. NAME OF MONITORING ORGANIZATION: Office of Naval Research
8a. NAME OF FUNDING/SPONSORING ORGANIZATION: Office of Naval Research	8b. OFFICE SYMBOL (If applicable):
8c. ADDRESS (City, State, and ZIP Code): 800 North Quincy Avenue, Arlington, Va 22217-5000	7b. ADDRESS (City, State, and ZIP Code): Code 1141, 800 North Quincy Ave, Arlington, VA 22217-5000
	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: N00014-81-C-0570
	10. SOURCE OF FUNDING NUMBERS: PROGRAM ELEMENT NO. 61153N; PROJECT NO. RR041-05; TASK NO. RR041-05-03; WORK UNIT ACCESSION NO NR204-123

11. TITLE (Include Security Classification): (U) LOCALIZING THE SUBUNIT POOL FOR THE TEMPORALLY REGULATED POLAR PILI OF CAULOBACTER CRESCENTUS

12. PERSONAL AUTHOR(S): Smit, John

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Summary Report	FROM 861001 TO 870930	1987	21

16. SUPPLEMENTARY NOTATION: In Press

17. COSATI CODES		
FIELD	GROUP	SUB-GROUP
06	03	

18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number): Keywords: Caulobacter crescentus, pili, Bacterium,

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

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20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT. ☐ DTIC USERS	UNCLASSIFIED

22a. NAME OF RESPONSIBLE INDIVIDUAL	22b. TELEPHONE (Include Area Code)	22c. OFFICE SYMBOL
Head, Biological Sciences Div, ONR	(202)696-4986	ONR Code 1141

DD FORM 1473, 84 MAR — 83 APR edition may be used until exhausted. All other editions are obsolete.

SECURITY CLASSIFICATION OF THIS PAGE

Caulobacter crescentus. Smit, John

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Revised sent 4.3-87

Localizing the Subunit Pool for the Temporally Regulated Polar Pili of Caulobacter crescentus

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Abstract

The pili of the stalked bacterium Caulobacter crescentus are assembled at a specific time in the life cycle at one pole of the cell and are composed of the monomer protein, pilin. A previous study demonstrated that the onset of pilin synthesis occurs well before pili appear on the surface, suggesting that pilin accumulates within the cell. In the present study an electron microscope immunocytochemistry assay was used to determine the subcellular location of this unassembled pilin and its fate during pilus assembly and cell division. Populations of synchronously growing cells were embedded in epoxy resin at selected times during the cell cycle. Ultrathin sections were treated with pilin-specific antibody, followed by protein A coupled to colloidal gold. It was determined that the cellular location for unassembled pilin was the cell cytoplasm. All cell membranes and regions of nuclear material were poorly labeled. Quantitation demonstrated that label density increased during the period of pilin synthesis and declined during the period of pilus assembly and maintenance. The pilin pool was not unequally segregated at division, for example, to the daughter cell that is elaborating pili. Mutants which have simultaneously lost the ability to produce flagella, pili and other polar organelles, possibly due to alterations in the specialized region of polar organelle assembly, were also examined by the immunocytochemistry technique. There was no significant difference in the pilin pool size relative to the wild type, indicating that pilin synthesis continues in the absence of a functioning assembly site. This pattern of synthesis and assembly for the pilus is significantly different from that of the polar flagellum which is produced at the same time and location on the cell surface. These findings are discussed in relation to the hypothesized organization center at the cell pole which may have a major role in directing the assembly of all the polar structures.

Introduction

During its life cycle the bacterium Caulobacter crescentus produces several surface structures at a specific location on the cell surface (1,2). Among the structures produced are a single flagellum, several pili, an adhesive substance (termed a holdfast) and a stalk, fashioned from the membranes that constitute the

cell wall of this gram-negative bacterium. In addition, these structures are expressed at a specific time during the life cycle; the flagellum, pili and holdfast appear simultaneously at the cell pole while the stalk is elaborated at the same position later in the cell cycle. Thus this bacterium provides a distinctive opportunity to study molecular details of gene regulation, morphogenesis and the mechanisms of directing the assembly of structures to specific sites in a cell.

The differentially-expressed structures are quite different from one another. The pilus filament is a relatively simple structure, composed of a single protein, pilin (3). Whether there is a basal structure containing additional components is unknown. The flagellum is more complex, containing, in addition to two filament proteins (4), a protein that polymerizes to form a hook (5,6) and numerous proteins that comprise the motor apparatus at the base of the flagellum (7). The adhesive holdfast is a polysaccharide (J. Smit, unpublished studies), which presumably requires the presence of several enzymes and activated substrates at the localized region of expression.

One approach in studying these structures is to address the complexity of temporal and spatial control of their coordinated assembly. That is, are the mechanisms of regulated synthesis and assembly similar for each of the polar organelles or is the cell capable of focussing diverse processes simultaneously at one site in the cell?

Analysis of pilus production was begun in a previous study in part to compare findings with what is known about production of the flagellum. Differences in the production of the two organelles were noted (8). The most significant difference was that synthesis of pilin begins much earlier in the cell cycle than the time of appearance of the assembled filament on the surface (Fig 1). It may be that a cycle of pilin synthesis is complete before pilus assembly begins. Thus a pool of pilin likely develops in the cell. This contrasts with the time of flagellin and flagellar hook protein synthesis, which occurs just before these proteins are assembled (5,9).

The objective of this study was to determine the cellular location of the pilin pool. Because pilin is a small (8000 m.w.) and hydrophobic molecule (3), problems with

non-specific adherence were anticipated in using standard methods to fractionate cell membranes and the cytoplasm in preparation for immunoassay. Consequently, an electron microscope immunocytochemical approach was developed which allowed direct examination of internal regions of the cell.

Materials and Methods

Bacterial Strains and Growth Conditions

Caulobacter crescentus CB15 (ATCC 19089) was used for all experiments. Cells were grown in a peptone-yeast extract medium (PYE) (10) or a glucose and salts minimal medium (HMG)(11) at 30°C. Synchronous populations of swarmer cells were prepared by the Ludox colloidal silica density gradient technique (12).

Mutants of C. crescentus that simultaneously lose the ability to produce flagella, pili and the receptors for bacteriophage which attach at the developmental pole, have been described (13-15). Such mutants in strain CB15 are produced by selecting cells that are resistant to bacteriophage øCbK, by plating bacteria in the presence of excessive numbers of bacteriophage. Most resistant strains no longer bind the bacteriophage and also lose the other polar organelles, except for the stalk. Such øCbK-resistant strains were then examined for motility and sensitivity to a pilus-specific bacteriophage (øCb5) (16). The mutants were also examined by negative stain electron microscopy for the presence of pili and flagella. Several non-motile mutants which produced no flagella or pili and were resistant to both bacteriophage were examined by the immunocytochemistry methods discussed below.

Preparation of Immunological Labeling Reagents

Antiserum to highly purified pilin protein was prepared in a rabbit in similar fashion to that reported previously (8). Sera was partially fractionated by ammonium sulfate precipitation of immunoglobulins (17). For most experiments antibody to pilin was further purified by passage through a column of DE-52 (Whatman) (DEAE-cellulose) at low ionic strength (17), which purified the IgG fraction of the antisera, and affinity chromatography. An affinity column was prepared by standard methods (18), coupling purified pilin to CNBr-activated Sepharose 4B

beads (Pharmacia). Ammonium sulfate fractionated anti-pilin antisera was applied to the column in 10 mM potassium phosphate (pH 7.2) and 150 mM NaCl (PBS). Bound antibody was eluted with 0.6 M acetic acid in 150mM NaCl. Collected fractions were monitored spectrophotometrically at 280 nm and pH was neutralized by addition of calibrated amounts of 2N Tris base. Antibody preparations were concentrated by dry dialysis against Ficoll 400 (Pharmacia) followed by dialysis against PBS.

Preparation of rabbit antibodies to the periodic surface array layer of C. crescentus CB15 and to whole cells was previously described (19, 20). The IgG fraction of antisera was prepared by ammonium sulfate precipitation and DE-52 chromatography. Control IgG was prepared from non-immune rabbit sera.

Protein A-colloidal gold conjugates were prepared by standard methods (21), using 5-6 nm diameter particles produced by phosphorus reduction of chloroauric acid. To prepare a colloidal gold/anti-pilin antibody conjugate, 12 ml of colloidal gold particles, prepared by phosphorus reduction of the same volume of 0.0125% chloroauric acid was mixed with 0.5 mg of ammonium sulfate-fractionated anti-pilin antisera. After 3 min, 0.6 ml of 1% polyethylene glycol, average molecular weight 20,000 (PEG-20M), was added . The preparation was centrifuged at 100,000xg for 75 min. The pellet was suspended with 350 µl of 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5 mg/ml PEG-20M and 3 mM NaN_3. This preparation was centrifuged at 15,000xg for 3 min to remove aggregates of colloidal gold particles.

Immunocytochemistry and Electron Microscopy Procedures

Label of the polar pili

Cells were grown in HMG medium to an optical density (660nm) of 0.35 and chilled on ice. 250 µl of cells were mixed with 75 µl of anti-pilin/colloidal gold conjugate and incubated 20 min on ice. To separate unbound conjugate from the cells, the mixture was applied to a 0.7 cm X 26 cm column of Sepharose 2B-Cl (Pharmacia), with water as eluant. Fractions were monitored by light microscopy for the presence of cells and visually for the red color of the unbound colloidal gold particles. The cell peak was concentrated by dialysis against dry Sephadex G200 (Pharmacia) beads. This method was developed to avoid centrifugation, which efficiently shears the gold particle-covered pili from the cells. The preparation

was viewed by electron microscopy as unstained whole mounts (Fig 2).

Fixation and Embedding for Immunocytochemistry

For fixation and embedding of unsynchronized cells, PYE medium was used for growth. HMG medium was used for growth of cells from which synchronous populations were obtained by the Ludox density gradient method. For fixation and embedding of synchronous populations, portions of the culture were removed at 0, 30, 60, 90 and 125 min. In all cases cells were pelleted by centrifugation and suspended in cold 3% formaldehyde (freshly prepared from paraformaldehyde), 0.1N sodium cacodylate buffer (pH 7.5), 5 mM $CaCl_2$ and 5 mM $MgCl_2$ for 1-1/2 hr on ice. The preparations were centrifuged and the cell pellets suspended with an equal volume of 2.5% agarose (Seakem ME, FMC Co.) dissolved in 0.1 M sodium cacodylate (pH 7.5), 5 mM $CaCl_2$ and 5 mM $MgCl_2$ (fixation buffer), held at 45°C. After cooling, the solidified cell pellets were cut into pieces and rinsed twice with fixation buffer, to which a small amount of basic fuchsin was added. The dye stains the agarose and assists visualizing the cell material after embedding. Dehydration was accomplished with a graded series of ethanol concentrations in water. The cell material was then transferred sequentially to ethanol:propylene oxide (1:1) for 10 min, propylene oxide for 10 min, propylene oxide:Araldite 502 resin (1:1) for 45 min, and Araldite resin for 4 hr. After transfer of cell material to molds, the resin was polymerized by treatment at 62°C for 24 hr.

Postembedding Label of Thin Sections

Ultrathin sections were cut and mounted on 300 mesh nickel grids that were made adhesive by dipping in a solution of benzene and Scotch transparent tape (3M Co.) (30 cm tape/50 ml benzene), followed by blotting off excess fluid. Most subsequent treatments were accomplished by floating the grids on droplets of reagents. The sections were treated with 10% hydrogen peroxide for 15 min and were rinsed by application of 20-40 ml of water to the grid (held by forceps) from a squirt bottle ("squirt wash"). After blotting the excess water, the grids were treated with PBS containing ovalbumin (10 mg/ml), D,L-lysine (1 mg/ml) and gelatin (1 mg/ml) (OLG), for 5 min. Grids were then transferred to droplets of antibody diluted in OLG. Affinity purified anti-pilin was used at 10 µg/ml. The IgG fractions of anti-whole cell and anti-surface array antibody were combined and used at a

concentration of 40 µg/ml each, as was control IgG. Grids were incubated overnight at 4°C followed by a squirt wash with 3.0 N NaCl and 10 mM potassium phosphate (pH 7.0) (high salt buffer). Grids were floated on the same buffer for an additional 10 min, followed by a PBS squirt wash and treatment with OLG for 5 min. Grids were transferred to protein A/colloidal gold conjugate, diluted 1:8 in OLG, and incubated 1 1/2 hr at room temperature. A squirt wash with high salt buffer and then water followed. The sections were then stained with 2.5% uranyl acetate and Reynold's lead citrate (22).

The colloidal gold labeling of thin-sectioned cells at each selected time during the course of growth of a synchronous population was quantitated. Micrographs were taken with a minimum amount of subject selection, to minimize bias, with the following exceptions; there was a bias toward regions of thin sections with as low a background label as possible and longnitudinal cross sections of cells were preferred, since more accurate quantitation was possible with larger cell areas. A replica grating grid was used for magnification calibration. Prints were produced from the micrographs at a calibrated magnification. An acetate overlay was made for each print and the outline of each cell selected for quantitation was traced twice on the overlay. One of the tracings was used to provide a record of the colloidal gold particles scored within the selected cell. The other tracing was overlaid in a nearby region of bare resin on the print and a count was made of the background non-specific label. This value was subtracted from the cell count value. The cross-sectional area of the selected cell was determined using the area calculation mode of an electronic coordinate digitizer (Numonics, model 1250). More than 40 μm^2 of exposed cell material was scored for both the anti-pilin label and the label with combined whole cell and surface layer antibodies. A total of approximately 60,000 colloidal gold particles were scored.

For plotting, the values calculated for each time point (as particles/μm^2) were multiplied by a factor corresponding to the increase in mass of the synchronous culture, assuming a mass doubling time of 125 min. Thus the plotted data is representative of the pilin pool of an entire synchronous culture and is comparable to a previous immunoassay method, which also determined pilin synthesis rates for a synchronous culture. Data were plotted with no further adjustment or after

normalization against the values obtained with the anti-whole cell/surface array antibody label, based on the assumption that the latter label may be an internal standard of uniform, non-cyclic synthesis of cell components.

RESULTS

Post-embedding Label of Thin Section

Unsynchronized Cell Populations

Treatment of thin sections with anti-pilin antibody and protein A/colloidal gold resulted in a characteristic labeling pattern. Only regions containing cytoplasm were labeled (Fig 3). There was no appreciable labeling of the inner or outer membrane, or mesosomal membranes within the cell. The cellular stalk was not labeled, nor were clear inclusions which are presumed to be poly-β-OH-butyrate (PHB), a storage polymer (10). Caulobacter does not exhibit a single compact region of nucleoid material. Instead, several regions are usually seen in thin section analysis; these regions were not appreciably labeled. All cells were labeled, although qualitatively there was some variability in label density. There was no indication of a gradient of labeling density within a cell, either from one end to the other or from the cell center toward the periphery.

In contrast to the anti-pilin results, use of antibodies directed to the whole cell resulted in label that was frequently associated with the cell membranes and nucleoid regions, in addition to the cytoplasm. Like the results with anti-pilin, PHB inclusions were not labeled. The density of label using anti-whole cell and surface array antibody was not significantly greater than that recorded for anti-pilin. This may mean that the label seen represents a minor fraction of cell components whose antigenic determinants remained intact after the fixation/embedding process (see below).

Treatment of thin sections with non-immune IgG resulted in very low levels of label within cells (Fig 4). Correction for non-specific background label usually resulted in a negative value; although all values were low (ranging from 10-30 particles/μm^2), apparently there is a slightly greater attraction for resin than for cell material.

As an additional negative control, an unrelated cell material, Trypanosoma brucei, a protozoan parasite, was fixed and embedded in similar fashion to the Caulobacter preparations. An attempt to label sections with anti-pilin antibody resulted in no label above background (data not shown).

Three independent øCbK-resistant mutants which produced no pili or flagella were also examined by the thin section labeling methods. All were positively labeled with pilin-specific antibody and the level of labeling was indistinguishable from non-mutant cells (Fig 5).

Synchronous Cell Populations

A distinct periodicity of label density was noted using the anti-pilin antibody (Table 1 and Fig 6). The lowest level of label density occured in the 30 min sample. Another drop in labeling density, relative to previous time points was noted in the 125 min sample. In the 125 min time point, stalked and swarmer cells are in equal numbers since division has just occured. These two cell types cannot be reliably distinguished in thin sections since most often, important morphological details, such as stalks, are not in the section plane. However, analysis of the quantitation data from this time point did not reveal two populations of cells distinguishable by label density. It is inferred then that there was no preferential distribution of pilin amongst the progeny during division.

The labeling pattern of the combined anti-whole cell/anti-surface array antibodies was not completely uniform during the cell cycle (Table 1). Although the 30, 60 and 90 min time points demonstrated similar label values, the 0 min time point was somewhat above average, while the 125 min time point was below average. The reason for this variation is not known, but may be related to the fact that it is not known which or how many of the cell components have retained antigenicity during the fixation and embedding process. Thus the value of this label as an internal measure of non-cyclic synthesis of cell components is suspect. For this reason the anti-pilin labeling data was plotted with and without normalization with this internal standard.

An ideal negative control for these studies would be the label of a Caulobacter

mutant producing no pilin. Such a mutant is not available, which places additional emphasis on the monospecificity of the affinity purified antipilin antibody. A previous immunoprecipitation assay with pilin antibody resulted in the coprecipitation of several other proteins which were developmentally regulated and thought to be physically associated with pilin at the developmental pole, such that antibody binding to one protein led to the coprecipitation of the others (8). Western blot analysis of both the affinity purified antipilin antibody and non-immune IgG used here (data not shown) leads to a faint label of numerous other cell proteins. This is a common problem experienced with the western blot assay, such that the method often cannot be used to definitively assess monospecificity of an antibody. Thus, current immunoassay methods cannot provide an alternate measure of monospecific binding to pilin. However, because of the high purity of the pilin protein (3) used to raise antibody and to affinity purify it, additional binding activities are expected to be, if not absent, a minor factor in the thin section labeling studies. Moreover all potential contaminating activities are directed to proteins that are not developmentally regulated or who temporal regulation is completely different from that of pilin (8). In both cases the effect would be to degrade the pattern of labeling seen in the current analysis, but not alter the conclusions.

DISCUSSION

Previous studies demonstrated that pilin is not made during the swarmer cell stage of the cell cycle (8), the stage at which the pili are present on the cell surface. Synthesis begins during the swarmer to stalked cell transition which occurs at 30-40 min for cells that divide in 120-125 min, and declines before division. Because pili do not appear on the cell until just before division (16), a pool of unassembled pilin was predicted.

Pilin is a protein with a high percentage (70%) of hydrophobic amino acids (3) and is assembled into a pilus at one discrete location on the cell surface. Thus, the finding that unassembled pilin accumulates in the cytoplasm and was not at all associated with any of the cell membranes prior to assembly was perhaps unexpected. Yet the results obtained by examining synchronous populations of cells with the post-embedding label method were in good agreement with the previous immunoassay method. It is concluded then that the cytoplasm is the location of the

pilus subunit protein pool.

The postembedding labeling for pilin showed that at the 30 min time point in a synchronous population, the apparent pool of unassembled pilin was at a minimum. The quantity of pilin increased after that time and then declined at the time of division. This is consistent with the expectation that the pilin pool is drawn upon for pilus assembly beginning just before division. Pilus assembly probably continues after division to lengthen the fragile pili or replace lost ones. As expected then, the pilin pool of the 0 min swarmer cells is larger than at the 30 min point in the cell cycle.

There was no indication that a concentration gradient forms within the cell or that at division there is a preferential accumulation of pilin in the swarmer cell progeny. It might be concluded that at division the capacity to <u>assemble</u> a pilus is segregated to the swarmer cell rather than the monomer protein needed to produce it.

In contrast to the segregration of the capacity to assemble a pilus, the <u>synthesis</u> of pilin appears to segregate at division in the opposite direction. Swarmer cells do not synthesize pilin and presumably the stalked cell progeny of a division immediately initiate another round of pilin synthesis. This segregation might be due to the separation of transcriptionally active and inactive pilin genes, although whether regulation of pilin synthesis is in fact at the level of transcription has not yet been resolved. DNA replication in Caulobacter is coordinated with morphological development (12), where a single round of chromosome replication occurs each generation and the progeny of a division each receive a copy. The physical properties and associated proteins of each chromosome are significantly different and it has been postulated that the differences may be a reflection of distinctive transcriptional as well as replication activities (23).

The observation that the pilin pool develops in mutants that do not assemble polar organelles (including pili) suggests that synthesis and assembly of pilin are not coordinated processes to the extent that failure to assemble a pilus does not inhibit continued pilin synthesis. This is not the case for the flagellar proteins. Synthesis of flagellins and the hook protein does not occur in the same type of polar

morphogenesis-defective mutants, i.e. those produced by selecting for resistance to polar bacteriophage (15).

The development of a monomer pool for a membrane protein or organelle that is stored within the cell prior to assembly has not been frequently reported for bacteria. Interestingly, most available information involves bacterial pili or fimbriae. There appears to be significant variety in the biogenesis of pili among various bacteria and types of pili produced. In E. coli, strains which express an F pilus accumulate the monomer protein for this organelle in the inner membrane prior to assembly (24). Pseudomonas aeruginosa, like Caulobacter, produces polar pili and a monomer pool of pilin accumulates. In contrast to Caulobacter, the location for this pilin pool is in the outer and inner membranes of the organism (25). With the K99 fimbriae of enterotoxigenic E. coli, synthesis and assembly of the fimbriae are separately regulated processes. The fimbriae subunits are produced in the inner membrane and transported to the periplasm, where they accumulate prior to the appropriate stimulus for assembly (26). To the best of our knowledge, the present study with Caulobacter pilin is the only known instance where the cytoplasm of the cell is the storage location for unassembled pilin.

The present study does not address in what form the pilin exists in the cytoplasm or how it is recruited to the developmental pole at the time of pilus assembly. Caulobacter pilin is hydrophobic, a factor which seems inappropriate in the aqueous environment of the cytoplasm. It is possible that the protein is able to fold in such a fashion that hydrophobic residues are not presented on the surface and that the protein goes through conformational changes during transport across the cell wall. Alternatively, the monomer may be specifically associated with another cell component which stabilizes pilin while in the cytoplasm. What information is present in the monomer and the polar region which enables recruitment of pilin to the pole and assembly of a pilus at a defined time in the life cycle is unknown. But because of the separation in synthesis and assembly of pilin, both temporally and spatially, this interaction may be more fundamental in accomplishing the regulated production of pili than temporal control of pilin synthesis.

The distinctive cell development or differentiation processes that characterize

Caulobacter all occur in a spatially restricted area of the cell wall, termed the polar region. It has been hypothesized by several groups (19, 27) that this region exists as a distinctive entity, both functionally and biochemically, which is produced and later activated at predictable times during the cell cycle. The mutants discussed above that are unable to express most polar organelles may have defects in the functioning of this region. The segregation of the capacity to assemble a pilus to the swarmer cell may well be because only the swarmer cell progeny has an active polar region. One reason for studying the mechanism of pilus production in Caulobacter is to compare findings with those obtained for the polar flagellum, since both structures are elaborated at the same time at the polar region. Such comparisons improve our understanding of the functional complexity of the polar region and its interaction with regulated genes and gene products. We have learned that the flagellum and pilus are produced and assembled in very different ways. The flagellins and the hook protein are made at the same time their assembly occurs (5,9). Although the flagellins do not appear to be transported across the cell wall cotranslationally (28, 29), they are probably synthesized very near the pole, establishing a transient monomer pool. In addition, a functional polar region is required for flagellin synthesis. These characteristics are in direct contrast to the findirgs with pilin.

The polar region of the cell wall also serves as the site for the production of the adhesive holdfast, which is a polysaccharide (J. Smit, unpublished results) and for the distinctive growth of the membranes to produce the tubular stalk (19, 30). It is becoming apparent that this region of the cell is functionally complex, with many different types of biogenesis activities focussed on this area in order to accomplish the distinctive life cycle of this bacterium.

ACKNOWLEDGEMENTS

I thank Nina Agabian, in whose laboratory at the University of Washington much of this work was done, for support, encouragement and helpful discussions during experimentation and for a reading of the manuscript. I also thank Frank McNally for a review of the manuscript. The technical assistance of Patricia Culley is acknowledged. This work was supported by grants from the National Institutes of Health (GM25527), the National Science Foundation (PCM-8021551), the Washington

SeaGrant Program and the Office of Naval Research (N00014-81-C-0570).

FIGURE LEGENDS

Figure 1. Life cycle of Caulobacter crescentus. At left, the swarmer cell exhibits a single flagellum, several polar pili and an adhesive holdfast. During subsequent cell differentiation the flagellum and pili are lost and a stalk develops. A new polar region develops at the pole opposite the stalk and just before division a flagellum, pili and the holdfast appear simultaneously. Following division the stalked cell directly begins the production of another swarmer cell, while the swarmer cell progeny must first develop into a stalked cell. The bar under the time line indicates when pilin is produced during the cell cycle, as determined by an immunoprecipitation assay (8). Thus, much or all of a cycle of pilin synthesis occurs before the organelle is assembled at the polar region.

Figure 2. A swarmer cell is shown labeled with anti-pilin antibody coupled to colloidal gold particles. Note the unlabeled polar flagellum. Bar indicates 0.2μm.

Figure 3. Postembedding label of C. crescentus CB15 with affinity-purified anti-pilin antibody and protein A-colloidal gold particles. The cytoplasm of this stalked cell appears uniformly labeled and there is little or no label associated with the inner and outer membranes or the stalk. Bar indicates 0.2μm.

Figure 4. Postembedding label using non-immune rabbit IgG. This example is typical of the low backgound obtained with non-specific antibody. Bar indicates 0.2μm.

Figure 5. Postembedding label of a C. crescentus CB15 mutant, K2-6, selected as resistant to bacteriophage φCbK. As discussed in the text, the receptor for this bacteriophage is located in the polar region and resistant mutants of this type are also defective in the production of other polar organelles, including pili and flagella. This cell is labeled with anti-pilin antibody, indicating that a reservoir of pilin is still produced in the absence of pilus assembly. Bar indicates 0.2μm.

Figure 6. Plotting the results of the quantitative analysis of anti-pilin/ protein A-colloidal gold postembedding label of a synchronously growing population of C. crescentus. As discussed in the text, the values calculated for each time point (as

particles/μm^2, see Table 1) were multiplied by a factor corresponding to the increase in mass of the synchronous culture and so represent the pilin pool of an entire synchronous culture. Additionally, the anti-pilin label results were plotted with (open circles) and without (closed circles) normalization to the results obtained using whole cell antibody.

Table 1. Results of quantitative postembedding labeling of a synchronously growing population of C. *crescentus*. As discussed in the text, the data has been corrected for non-specific background label. Data for non-immune IgG label was also determined at each time point, but was always zero or slightly negative and is not shown here. The values in parentheses indicate the 95% confidence interval determined by standard statistical methods.

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Tab 1

Time Point in Synchrony	Pilin Antibody			Whole Cell Antibody		
	μm^2 scored	Particles (P)	$P/\mu m^2$	μm^2 scored	Particles (P)	$P/\mu m^2$
0 min	6.85	5605	819 (±34)	8.57	6394	746 (±20)
30 min	6.81	3202	470 (±20)	9.25	5935	642 (±15)
60 min	9.47	7241	764 (±17)	9.65	6565	680 (±14)
90 min	8.58	6762	788 (±21)	8.42	5816	691 (±19)
125 min	9.66	5607	580 (±12)	10.58	6010	568 (±10)

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