Cell Cycle Arrest of a *Caulobacter crescentus secA* Mutant

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Cell differentiation is an inherent component of the *Caulobacter crescentus* cell cycle. The transition of a swarmer cell, with a single polar flagellum, into a sessile stalked cell includes several morphogenetic events. These include the release of the flagellum and pili, the proteolysis of chemotaxis proteins, the biogenesis of the polar stalk, and the initiation of DNA replication. We have isolated a group of temperature-sensitive mutants that are unable to complete this process at the restrictive temperature. We show here that one of these strains has a mutation in a homolog of the *Escherichia coli secA* gene, whose product is involved in protein translocation at the cell membrane. This *C. crescentus secA* mutant has allowed the identification of morphogenetic events in the swarmer-to-stalked cell transition that require SecA-dependent protein translocation. Upon shift to the nonpermissive temperature, the mutant *secA* swarmer cell is able to release the polar flagellum, degrade chemoreceptors, and initiate DNA replication, but it is unable to form a stalk, complete DNA replication, or carry out cell division. At the nonpermissive temperature, the cell cycle blocks prior to the de novo synthesis of flagella and chemotaxis proteins that normally occurs in the predivisional cell. Although interactions between the chromosome and the cytoplasmic membrane are believed to be a functional component of the temporal regulation of DNA replication, the ability of this *secA* mutant to initiate replication at the nonpermissive temperature suggests that SecA-dependent events are not involved in this process. However, both cell division and stalk formation, which is analogous to a polar division event, require SecA function.

Caulobacter crescentus is a gram-negative bacterium that divides asymmetrically to produce two different types of cells: a motile swarmer cell and a sessile stalked cell (see Fig. 9) (13, 24). These cell types differ not only in their cell morphologies but also in their developmental programs. While the progeny stalked cell immediately initiates DNA replication and subsequent cell division, the swarmer cell cannot undergo DNA replication and cell division until it differentiates into a stalked cell.

The swarmer-to-stalked cell differentiation is a multistep process. The flagellum of a swarmer cell is shed, and a stalk is formed at the site previously occupied by the flagellum (35). Several chemotaxis proteins are specifically degraded at this stage by a temporally controlled proteolytic activity, which may also contribute to the concomitant release of the flagellum (2, 14). In addition, the sedimentation coefficient of the quiescent swarmer cell chromosome dramatically decreases as the swarmer cell differentiates into the stalked cell (9, 13, 41). An unknown cue signals the initiation of DNA replication in the stalked cell (3, 20). This swarmer-to-stalked cell transition provides a tractable system with which to study the factors that trigger cellular differentiation events and the initiation of chromosome replication. A number of temperature-sensitive (ts) cell cycle mutants of *C. crescentus* have been previously isolated and analyzed (28, 29, 38). The majority of these mutants exhibit defects either in DNA replication or in the cell division process, although the affected gene has been identified in only two instances (25, 42).

In an effort to identify the genes that mediate the differentiation of the swarmer cell into a stalked cell, we have isolated and characterized ts mutants that arrest the cell cycle at the nonpermissive temperature but remain viable. Analysis of one of these mutants revealed that protein translocation mediated

by SecA is required for stalk biogenesis and cell division but is not required for the initiation of chromosome replication at the swarmer-to-stalked cell transition stage of the cell cycle.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. The bacterial strains and plasmids used in this study are listed in Table 1. *C. crescentus* strains were grown at 23 or 30°C in either PYE medium (30) or minimal M2 glucose (M2G) medium (17). The nonpermissive temperature for ts mutants was 37°C. Plasmids were transferred from *Escherichia coli* to *C. crescentus* by conjugal transfer (7).

Isolation of ts mutants. ts mutants were isolated from *C. crescentus* LS107; this strain contains a deletion of the β-lactamase gene in the synchronizable strain NA1000 (previously called CB15N) (9). After mutagenesis with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine to produce 10% survival, mutagenized cells were allowed to recover in PYE medium at 23°C for one generation and then incubated overnight in PYE medium at 37°C in the presence of cephalosporin C (60 μg/ml). The treatment with cephalosporin C selects for cells which cannot grow at 37°C. The surviving cells were then plated on PYE plates and allowed to form colonies at 23°C. These colonies were patched on PYE plates and incubated at 23 and 37°C to identify ts mutants. Mutant cells grew normally at 30°C.

Screening of cell cycle-arrested mutants. ts mutants were grown at 23°C to early logarithmic phase in PYE medium and were then shifted to 37°C. Four to six hours after the temperature shift, the cells were examined by phase-contrast light microscopy. Mutant strains that appeared to arrest at a specific cell type were subjected to a secondary microscopic screening using synchronized cultures (9).

Cloning and mapping the *secA* gene. A cosmid library of *C. crescentus* genomic DNA (1) was introduced into the ts strain LS416 by mating and screened for temperature-resistant growth at 37°C on PYE plates. A complementing DNA fragment was identified, and its chromosomal map position

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description	Reference or source
Strains		
E. coli S17-1	E. coli 294::RP4-2(Tc::Mu) (Km::Tn7)	37
C. crescentus		
CB15	Wild type	30
NA1000	Synchronizable derivative of CB15	9
LS107	NA1000, Δbla	M. R. K. Alley
LS416	LS107, secA1	This study
Plasmids		
pRK290	Tc^r, IncP1 replicon, mob$^+$	6
pRK290-20R	pIC20R polylinker inserted into the EcoRI site of pRK290	12
pRK2525	RP4, Ap^r, Km^r, Tc::lacZ	P. Goncharoff
pJK400	pLAFR5-derived cosmid containing secA	This study
pJK401	The 2.6-kb BamHI fragment of pJK400 in pBR322	This study

was determined by Southern blot analysis of genomic DNA by using pulse-field gel electrophoresis as described previously (8).

DNA sequencing. The nucleotide sequence of the 3.2-kb BglII-SacI DNA fragment (Fig. 1) was determined by the dideoxy-chain termination method (34), using 7-deaza-dGTP (22) to reduce compression artifacts. M13mp18 and M13mp19 (44) were used as sequencing vectors.

Analysis of protein secretion defects. C. crescentus LS416 and its parental strain LS107, carrying RP4-derived plasmid pRK2525, were grown in M2 glucose minimal medium containing ampicillin (10 μg/ml) at 30°C to early logarithmic phase. Cultures (1 ml) were incubated at 30 or 37°C for 5 min before addition of 15 μCi of L-[^{35}S]methionine. Cells were then incubated in the presence of label for 5 min, harvested, and boiled in 50 μl of cell lysis buffer (2% sodium dodecyl sulfate [SDS], 50 mM Tris-HCl [pH 7.5]) for 3 min. Cell extracts were immunoprecipitated with anti-β-lactamase antibody (from 5' to 3' Inc.) as described previously (14).

Cell cycle immunoblot analysis. Swarmer cells were isolated by Ludox density gradient centrifugation (9) from cultures of LS416 grown in PYE medium at 30°C and were then allowed to proceed through the cell cycle at either 30 or 37°C. At 15- to 20-min intervals, 0.2 ml of synchronous culture was mixed with

FIG. 1. Restriction map of the C. crescentus secA gene region. The bar above the restriction map indicates the region, BglII to SacI, whose nucleotide sequence is shown in Fig. 2. The location of the secA coding sequence is shown below the restriction map.

the same volume of 2× gel sample buffer (4% SDS, 100 mM Tris-HCl [pH 6.8], 20% glycerol, 10% β-mercaptoethanol, 0.4% bromophenol blue). Samples were boiled for 3 min, and identical volumes of cell lysates were applied to an SDS-polyacrylamide gel (7.5%, wt/vol). Immunoblot analysis was carried out as described previously (2), using anti-McpA antiserum (a gift from M. R. K. Alley).

Cell cycle immunoprecipitations. Swarmer cells were isolated from cultures of strain LS416 or strain LS107 that were grown at 30°C and were then allowed to proceed through the cell cycle at either 30 or 37°C. At 20-min intervals, 1-ml aliquots of cells were pulse-labeled with 15 μCi of L-[^{35}S]methionine for 5 min. Labeled cells were harvested and boiled in 50 μl of cell lysis buffer for 3 min. Immunoprecipitation was done using anti-McpA and antiflagellin antisera as described previously (14).

Fluorescence-activated cell sorter (FACS) analysis. Swarmer cells of C. crescentus LS416 were isolated from cultures grown in PYE medium at 30°C and were then allowed to proceed through the cell cycle at either 30 or 37°C. At 0 and 150 min, cells were fixed with 70% ethanol and stored on ice for 1 h. The cells were then collected by centrifugation in microcentrifuge tubes at 8,000 rpm, resuspended in TMS buffer (10 mM Tri-HCl [pH 7.2], 1.5 mM MgCl$_2$, 150 mM NaCl) containing chromomycin A3 (10 μg/ml), and kept at 4°C in the dark overnight. Cells were analyzed in a Becton Dickinson FACStar Plus machine. The excitation wavelength was 458 nm, and fluorescence was measured at 495 nm.

Electron microscopy. Cells were fixed with 1% glutaraldehyde, incubated for 1 h on ice, and spotted onto Formvar-coated electron microscope grids. The samples were stained with 1% uranyl acetate for 30 s, washed with water, and examined on a Philips 300 electron microscope at 80 kV.

Nucleotide sequence accession number. The nucleotide sequence of the C. crescentus secA gene has been submitted to the GenBank database and assigned accession number U06928.

RESULTS

Isolation of ts mutants defective in cell differentiation. ts mutants were isolated that were not lethal, in that they resumed growth and division when shifted to the permissive temperature. These mutants arrested at specific stages of the cell cycle at the nonpermissive temperature. The mutant screen was facilitated by the classical β-lactam drug enrichment technique that selects for mutants that are unable to grow, but maintain viability at the nonpermissive temperature by killing actively growing cells. Mutagenized cells were treated with cephalosporin C at 37°C and then plated at 23°C in the absence of the drug. Resulting colonies were screened for ts growth at 37°C, and cells were examined by light and electron microscopy at both the permissive and nonpermissive temperatures. Among 60 independent ts mutants, 6 appeared to arrest at a specific stage of the cell cycle upon a shift to the nonpermissive temperature. One of these mutants, LS416, was able to release the flagellum and elongate during the swarmer-to-stalked cell transition but was unable to assemble a stalk or divide at the nonpermissive temperature.

Identification and isolation of the C. crescentus secA gene. To identify the mutant locus that resulted in the bacteriostatic cell cycle block of strain LS416, two cosmid clones were isolated by correction of the ts growth phenotype of strain LS416. To locate the gene within the C. crescentus DNA insert of the cosmids that corrected the ts growth, various DNA restriction fragments were subcloned into the broad-host-range plasmid
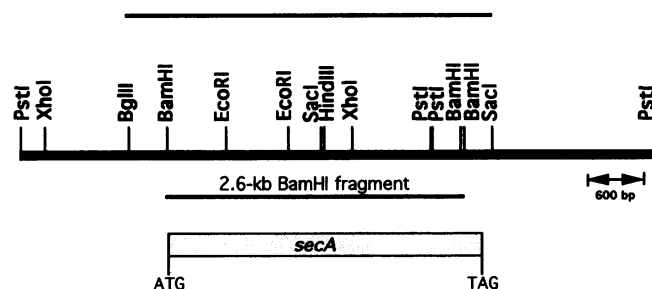
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   1  AGATCTCCGGCTTTGCTGGAATGGGCTCTAGCGCGGTTTTTACCCAAGGGGAACAGTTCGCCGTCGAAAGCGTTCTGCTCAGCGAAAACCGGCCCCGGAGCCAGGGTTTCAGCCCCTTAA
 121  ACCCTCGCTGTTCTAGGGTCTTCGCGTTGACACCAGGGGGGCCGGTGCTTAAGTCTCCGGCCGCACCGGAACAGCGGATTTATTCGTTGTTGCGGCCGATTTTCATCTCCGTAGTCGAGG
 241  CCCTCCAGCCTTAGCGCCGGCCTCCTCGTCCAAACCGCCCTCCGGATCCCCCCTGAATGCTTGGTTTCGCCAAAAAGCTCTTCGGCTCTTCCAACGAACGCAAGGTCAAGACCCTGGCCA
                                                                             M  L  G  F  A  K  K  L  F  G  S  S  N  E  R  K  V  K  T  L  A
 361  CGCGCGTGGCAAGATCAACGCCTATGAAGCCGAATACCGCCCTCTCCGACGAGGCGCTGAAGGGCAAGACGGCCGAGTTCAAGGCGCGTCTGGAAAAGGGCGAGACCCTCGACGACATTC
        T  R  V  A  R  S  T  P  M  K  P  N  T  A  L  S  D  E  A  L  K  G  K  T  A  E  F  K  A  R  L  E  K  G  E  T  L  D  D  I
 481  TCAACGAAGCCTTCGCCGTGGTGCGCGAGGCCTCCAAGCGCGTGCTGGGCATGCGCCACTTCGACGTCCAGATGGTGGGCGGCATGGTGCTGCACTTCTCGGGCATCTCGGAGATGCGCA
        L  N  E  A  F  A  V  V  R  E  A  S  K  R  V  L  G  M  R  H  F  D  V  Q  M  V  G  G  M  V  L  H  F  S  G  I  S  E  M  R
 601  CCGGTGAAGGCAAGACCCTGGTCGCCACCCTGCCGACCTATCTGAACGCCCTGGAAGGCAAGGGCGTCCACGTCATCACCGTCAACGACTATCTGGCCCGCCGCGACGCCGACTGGATGG
        T  G  E  G  K  T  L  V  A  T  L  P  T  Y  L  N  A  L  E  G  K  G  V  H  V  I  T  V  N  D  Y  L  A  R  R  D  A  D  W  M
 721  GTCAGGTCTACAACTTCCTGGGCCTGAGCTACGGCGTCATCGTCAACGGCCTGAGCCAAGGCGAGCGTCAGCGCGCCTATCGCAGCGACATCACCTACGGCACCAACAACGAATTCGGCT
        G  Q  V  Y  N  F  L  G  L  S  Y  G  V  I  V  N  G  L  S  Q  G  E  R  Q  R  A  Y  R  S  D  I  T  Y  G  T  N  N  E  F  G
 841  TCGACTATCTGCGCGACAACCTCGTCTACAGCGTCGACGAGATGGTCCAGCGCGGCCACAACTTCGCGATCGTCGACGAAGTGGACTCGATCCTGATCGACGAGGCCCGCACGCCGCTGA
        F  D  Y  L  R  D  N  L  V  Y  S  V  D  E  M  V  Q  R  G  H  N  F  A  I  V  D  E  V  D  S  I  L  I  D  E  A  R  T  P  L
 961  TCATCTCGGGCCCGACCGAGGACCGCAGCAGCTTCTACAAGACCATCGACGTGCTGGTTAAGGAGCTGATCCTCGACAAGTCGATGTTCGACCACGACGAGAAGCAAAGCAGGTCATCC
        I  I  S  G  P  T  E  D  R  S  S  F  Y  K  T  I  D  V  L  V  K  E  L  I  L  D  K  S  M  F  D  H  D  E  K  Q  K  Q  V  I
1081  TGACCGAAGACGGCCAGGAGAAGATCGAAGAGATCCTGATGTCGGCGGCCAACCTGGCCGAGGACTCGGCCGGTCTCTATCGACGCGCCAACGTCTCGGTGGTGCACCACGTCAATCAGG
        L  T  E  D  G  Q  E  K  I  E  E  I  L  M  S  A  A  N  L  A  E  D  S  A  G  L  Y  R  R  A  N  V  S  V  V  H  H  V  N  Q
1201  CCCTGCGCGCCAACATCCTGATCACGCGCGACAAGGACTACATCGTCAAGGGCGGCGAGGTTGTCCTGATCGACGAGTTCACCGGCCGCATGATGACCGGCCGCCGCCTGTCGGAAGGCC
        A  L  R  A  N  I  L  I  T  R  D  K  D  Y  I  V  K  G  G  E  V  V  L  I  D  E  F  T  G  R  M  M  T  G  R  R  L  S  E  G
1321  TGCACCAGGCCATCGAGGCCAAGGAAGGCGCCGACATCCAGCCCGAGAACCAGACCCTGGCCTCGGTGACCATCCAGAACTACTTCCGCCTCTACAAGAAGCTGTCGGGCATGACCGGCA
        L  H  Q  A  I  E  A  K  E  G  A  D  I  Q  P  E  N  Q  T  L  A  S  V  T  I  Q  N  Y  F  R  L  Y  K  K  L  S  G  M  T  G
1441  CCGCGTCGACCGAGGCCCAGGAATTCGACGACATCTACAAGATGAGCGTCTCGGAAATTCCGACCAACCGCACCATCCAGCGCATCGACGACGACGACGAGGTCTATCGCACCGAGCGCG
        T  A  S  T  E  A  Q  E  F  D  D  I  Y  K  M  S  V  S  E  I  P  T  N  R  T  I  Q  R  I  D  D  D  D  E  V  Y  R  T  E  R
1561  AGAAGAACGAGGCCATCCTCAAGCAGATCGCCGACTGCCACGTGCGCGGCCAGCCGATCCTGGTCGGCACCGTCTCGATCGAGAAGTCCGAAGAGCTCTCCAAGCTTCTGTCGACCTTCT
        E  K  N  E  A  I  L  K  Q  I  A  D  C  H  V  R  G  Q  P  I  L  V  G  T  V  S  I  E  K  S  E  E  L  S  K  L  L  S  T  F
1681  CCTTCGAGAAGGACGGCAAGAAGGTGAAGGGCATCCCCCACCAGGTGCTGAACGCCCGCTTCCACGAGCAGGAAGCCGTGATCGTCGCCGACGCGGGCGTGCCCGGCGGCGGTGACCATCG
        S  F  E  K  D  G  K  K  V  K  G  I  P  H  Q  V  L  N  A  R  F  H  E  Q  E  A  V  I  V  A  D  A  G  V  P  G  A  V  T  I
1801  CCACCAACATGGCCGGCCGCGGCACCGACATCCAGCTGGGCGGCAGCATCGACATCGCGCTGTTCAACTGGCGCCACGAGCAGCGCGGCATGGGTCTGGAGATCACCGTCGAGGACGAGG
        A  T  N  M  A  G  R  G  T  D  I  Q  L  G  G  S  I  D  I  A  L  F  N  W  R  H  E  Q  R  G  M  G  L  E  I  T  V  E  D  E
1921  CCGAAGAGCGCGCCCGCCTCGAGACCGAGATCGCCGACAAGAAGGCCCAGGCCCTGGCCGCCGGCGGCCTGTTCGTGCTGGGCACCGAGCGTCACGAAAGCCGCCGCATCGACAACCAGC
        A  E  E  R  A  R  L  E  T  E  I  A  D  K  K  A  Q  A  L  A  A  G  G  L  F  V  L  G  T  E  R  H  E  S  R  R  I  D  N  Q
2041  TGCGCGGCCGCACCGGCCGTCAGGGCGACCGGTCGTCGAAGTTCTTCCTGTCGTGCGAAGACGACCTGCTGCGCATCTTCGCCGGCGAGCGCTTGGATGCGATCATGCGCACCTTCGGCG
        L  R  G  R  T  G  R  Q  G  D  R  S  S  K  F  F  L  S  C  E  D  D  L  L  R  I  F  A  G  E  R  L  D  A  I  M  R  T  F  G
2161  TCCAGGAAGGCGAAGCCATCACCCACAAGTGGCTGAACAACGCCATCGCCACGGCGCAAAAGCGCGTCGAGCAGCGCAACTACGAAATCCGCAAGAACCTCCTGAAGTACGACGACGTCG
        V  Q  E  G  E  A  I  T  H  K  W  L  N  N  A  I  A  T  A  Q  K  R  V  E  Q  R  N  Y  E  I  R  K  N  L  L  K  Y  D  D  V
2281  TCAACGACCAGCGCAAGGCCGTGTTCGAGCAGCGCCAGGAGTTCATGGAGTCCAGCGACCTCTCGGACATCATCCACGAGATGCGTCGCGACGTGATCGACGACCTGGTCCTGCGCCACC
        V  N  D  Q  R  K  A  V  F  E  Q  R  Q  E  F  M  E  S  S  D  L  S  D  I  I  H  E  M  R  R  D  V  I  D  D  L  V  L  R  H
2401  TGCCGCCCAAGGCCTATGCCGAGCAGTGGGACGTCGAGGGCGTGACCGAGCGCGTCAAGTCTCTGGGTCTGGACCTGCCGATCGCCGAGTGGGCCGCCGAGGAAGGCATCGCCGACG
        L  P  P  K  A  Y  A  E  Q  W  D  V  E  G  L  T  E  R  V  K  S  I  L  G  L  D  L  P  I  A  E  W  A  A  E  E  G  I  A  D
2521  AGGAGATGAAAGAGCGCATCACCAAGGCGGCCGACGAATACGCGGCCCAGCGCGAGGTGATCATCACGCCCGAGCAGATGCGCTCGGTCGAGAAGAGCTTCCTACTGCAGATGATCGATC
        E  E  M  K  E  R  I  T  K  A  A  D  E  Y  A  A  Q  R  E  V  I  I  T  P  E  Q  M  R  S  V  E  K  S  F  L  L  Q  M  I  D
2641  TGCAGTGGCGCGAGCACCTGATGCACCTGGATCACCTGCGCAACGTCATCGGCCTGCGCGGCTATGGCCAGCGTGATCCGCTGAACGAGTACAAGACCGAGGCCTTCTCGCTGTTCGAGA
        L  Q  W  R  E  H  L  M  H  L  D  H  L  R  N  V  I  G  L  R  G  Y  G  Q  R  D  P  L  N  E  Y  K  T  E  A  F  S  L  F  E
2761  AGCTCTTGGGCGATCTGCGCACCAACACCACCCGCTGGCTGATGACGGTCGAGATCGCCTATGCCGAGCCGGAGGTCCCGCACACGCCGCTGGACAACCTGGTCGAGGTGCACCTGGATC
        K  L  L  G  D  L  R  T  N  T  T  R  W  L  M  T  V  E  I  A  Y  A  E  P  E  V  P  H  T  P  L  D  N  L  V  E  V  H  L  D
2881  CGCTGACCGGCGAGAACGCCGCCTTCGCCGGCGGGATCCCGGAGGGCGTGTCGACCGCCCAGCGCGAAGCCCTGCCCGTCTCGGCCCTGCCGGAAGGCTGGGACCGCACCAACCGCAACG
        P  L  T  G  E  N  A  A  F  A  G  G  I  P  E  G  L  S  T  A  Q  R  E  A  L  P  V  S  A  L  P  E  G  W  D  R  T  N  R  N
3001  CCCCTTGCCCCTGCGGCTCGGGCAAGAAGTTCAAGCAGTGCCACGGCTCGCTGGTGCGCTAGACGTCTAGCATCCGATCTCCCCGGCGAACGCGTCCAGATCGAACAGAAGGGCGCCGAG
        A  P  C  P  C  G  S  G  K  K  F  K  Q  C  H  G  S  L  V  R  *
3121  CGGCTTCGCTCGTTTCGCTTTCATGGCGGACGCTCAAGGATGGATCTGGGCTTCGGCGTTCGCTGAGGAGCTC   3193
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FIG. 2. Nucleotide and predicted amino acid sequences of the *C. crescentus secA* gene. The nucleotide sequence is shown from the *BglII* site to the *SacI* site (see Fig. 1). The putative ribosome binding site is underlined.

pRK290-20R (12) and tested for the ability to allow growth of strain LS416 (a *rec+* strain) at 37°C. A 2.6-kb *BamHI* fragment (Fig. 1), common to both complementing cosmids, was found to rescue the ts growth of strain LS416.

The position of the complementing DNA fragment on the physical map of the *C. crescentus* chromosome was determined by Southern blot analysis after pulse-field gel electrophoresis of chromosomal DNA digested with *AseI*, *DraI*, and *SpeI*. The 2.6-kb *BamHI* fragment hybridized to a 420-kb *AseI*, a 240-kb *DraI*, and a 250-kb *SpeI* fragment (data not shown). Comparison of the physical and genetic maps (8) allowed us to determine that the 2.6-kb *BamHI* fragment maps approximately 200 kb clockwise from the *aroC* locus.

The 2.6-kb *BamHI* fragment was within a *BglII*-to-*SacI* restriction fragment that was sequenced on both strands (Fig. 2). Analysis of the DNA sequence revealed a long open reading frame whose predicted amino acid sequence had extensive homology to the *E. coli* SecA protein sequence (Fig. 3). Rescue of the ts growth of LS416 by the *BamHI* fragment presumably occurred via homologous recombination, because the 2.6-kb *BamHI* fragment lacked 59 amino acid residues that are present at the C-terminal end of *E. coli* SecA. However, the *BglII*-to-*SacI* restriction fragment contained the entire *secA* homolog encoding a polypeptide of 921 amino acid residues with a molecular mass of 106 kDa. The predicted *C. crescentus* SecA protein shows 53.5% amino acid sequence identity and 72% similarity to the *E. coli* SecA protein (Fig. 3).

Since the *E. coli* SecA protein is a major component of the

protein secretion process (26), we examined strain LS416 to determine if it had a defect in protein secretion. Protein secretion was assayed by determining the accumulation of the precursor protein of β-lactamase, a periplasmic protein whose secretion is known to be SecA dependent in *E. coli* (19). Wild-type and LS416 *C. crescentus* cultures, both with an RP4-derived plasmid (pRK2525) carrying the β-lactamase gene, were incubated at 30 or 37°C for 5 min before being labeled for 5 min with L-[35S]methionine (Fig. 4). Although wild-type cells also showed some accumulation of precursor protein at 37°C, the *C. crescentus secA* mutant strain LS416 had a severe defect in the secretion of β-lactamase precursor protein after only 5 min of preincubation at the nonpermissive temperature. This result, together with the sequence homology to the *E. coli* SecA protein, clearly shows that the complementing gene is a *C. crescentus* homolog of the *E. coli secA* gene.

The swarmer-to-stalked cell transition is aberrant in the *C. crescentus secA* mutant. To characterize the cell cycle arrest phenotype of the *secA* mutant strain LS416, swarmer cells were isolated from wild-type and mutant cultures grown at 30°C. These swarmer cells were then shifted to 37°C and allowed to proceed synchronously through the cell cycle. Progression through the cell cycle at 37°C of both the wild-type strain, LS107 (Fig. 5A), and mutant strain LS416 (Fig. 5B) was monitored by electron microscopy. Schematics of the cell cycle are shown below the micrographs. The parent strain LS107 progressed normally through the cell cycle at 37°C, but the mutant cells appeared to arrest as elongated cells with no

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  1 MLIKLLTKVFGSRNDRTLRRMRKVVNIINAMEPEMEKLSDEELKGKTAEF 50
    :: : .I:III.I:I.:: : . I.  .:I.:: . IIII.IIIIIIII
  1 .MLGFAKKLFGSSNERKVKTLATRVARSTPMKPNTA.LSDEALKGKTAEF 48

 51 RARLEKGEVLENLIPEAFAVVREASKRVFGMRHFDVQLLGGMVLNERCIA 100
    :IIIIIII.I:::: IIIIIIIIIIIIIIII::IIIII: ...I.
 49 KARLEKGETLDDILNEAFAVVREASKRVLGMRHFDVQMVGGMVLHFSGIS 98

101 EMRTGEGKTLTATLPAYLNALTGKGVHVVTVNDYLAQRDAENNRPLFEFL 150
    IIIIIIIIII.IIII.IIIII.IIIIII:IIIIIII.III:  .:::II
 99 EMRTGEGKTLVATLPTYLNALEGKGVHVITVNDYLARRDADWMGQVYNFL 148

151 GLTVGINLPGMPAPAKREAYAADITYGTNNEYGFDYLRDNMAFSPEERVQ 200
    II. I: : I:.....:. II .IIIIIIIII:IIIIIIII::I.:I.II
149 GLSYGVIVNGLSQGERQRAYRSDITYGTNNEFGFDYLRDNLVYSVDEMVQ 198

201 RKLHYALVDEVDSILIDEARTPLIISGPAEDSSEMYKRVNKIIPHLIRQE 250
    I  ::I:IIIIIIIIIIIIIIII IIIIIIII:.II.I.:II :: ::..II
199 RGHNFAIVDEVDSILIDEARTPLIISGPTEDRSSFYKTIDVLVKELI... 245

251 KEDSETFQGEGHFSVDEKSRQVNLTERGLVLIEELLVKEGIM.DEGESLY 299
    :.: I. III :II III I   III:I:..: : :::.:II
246 .......LDKSMFDHDEKQKQVILTEDGQEKIEEILMSAANLAEDSAGLY 288

300 SPANIMLMHHVTAALRAHALFTRDVDYIVKDGEVIIVDEHTGRTMQGRRW 349
    ..II: ::III..IIII: I:III IIIII:III::II III I III:
289 RRANVSVVHHVNQALRANILITRDKDYIVKGGEVVLIDEFTGRMMTGRRL 338

350 SDGLHQAVEAKEGVQIQNENQTLASITFQNYFRLYEKLAGMTGTADTEAF 399
    I:IIIII:IIII.:II IIIIIII:I:IIIIII.II.IIIIII.III
339 SEGLHQAIEAKEGADIQPENQTLASVTIQNYFRLYKKLSGMTGTASTEAQ 388

400 EFSSIYKLDTVVVPTNRPMIRKDLPDLVYMTEAEKIQAIIEDIKERTAKG 449
    II..III:.. :IIII.: I I .I II.II II :III::.I : .III
389 EFDDIYKMSVSEIPTNRTIQRIDDDDEVYRTEREKNEAILKQIADCHVRG 438

450 QPVLVGTISIEKSELVSNELTK.........AGIKHNVLNAKFHANEAA 489
    II:IIIII:IIIIII :I. I..       II.I.IIII:III.:III
439 QPILVGTVSIEKSEELSKLLSTFSFEKDGKKVKGIPHQVLNARFHEQEAV 488

490 IVAQAGYPAAVTIATNMAGRGTDIVLGGS...........WQAEVAALE 527
    III:IK I:IIIIIIIIIIIIIII IIII        :. ::.
489 IVADAGVPGAVTIATNMAGRGTDIQLGGSIDIALFNWRHEQRGMGLEITV 538

528 NPTAEQIEKIKADWQVRHDAVLEAGGLHIIGTERHESRRIDNQLRGRSGR 577
    :..II: .::..: . :.....I.IIII ::IIIIIIIIIIIIIII.II
539 EDEAEERARLETEIADKKAQALAAGGLFVLGTERHESRRIDNQLRGRTGR 588

578 QGDAGSSRFYLSMEDALMRIFASDRVSGMMRKLGMKPGEAIEHPWVTKAI 627
    III  II:I:II II.I:IIII::I::::I.:::.IIII.I.I:..II
589 QGD.RSSKFFLSCEDDLLRIFAGERLDAIMRTFGVQEGEAITHKWLMNAI 637

628 ANAQRKVESRNFDIRKQLLEYDDVANDQRRAIYSQRNELLDVSDVSETIN 677
    I.II::II II::III.II.IIII.II:::I :.I::.I II:I:.:
638 ATAQKRVEQRNYEIRKNLLKYDDVVNDQRRAVFEQRQEFMESSDLSDIIH 687

678 SIREDVFKATIDAYIPPQSLEEMWDIPGLQERLKNDFDLDLPIAEWLDKE 727
    ..:I II:.. : .:II....I II:.II II:I. ::IIIIIII ...I
688 EMRRDVIDDLVLRHLPPKAYAEQWDVEGLTERVKSILGLDLPIAEWAAEI 737

728 PELHEETLRDGILAQSIEVYQRKEEVVGAEMMRHFEKGVMLQTLDSLWKE 777
    :: :I.::: I  .. I  ..:I ::.:I II .II::II :I I:I
738 .GIADEEMKERITKAADEYAAQREVIITPEQMRSVEKSFLLQMIDLQWRE 786

778 HLAAMDYLRQGIHLRGYAQKDPKQEYKRESFSMFAAMLESLKYEVISTLS 827
    II :I.II..I IIII:I:II .III I.II:I. :I:.I: :.... I
787 HLMHLDHLRNVIGLRGYGQRDPLNEYKTEAFSLFEKLLGDLRTNTTRWLM 836

828 KVQVRMPE.EVEELEQQRRMEAERLAQMQQLSHQDDDSAAAAALAAQTGE 876
    .I:: :I II.. :. :I   .:::.I. ::.. I:: : I::
837 TVEIAYAEPEVPHTPLDNLVE....VHLDPLTGENAAFAGGIPEGLSTAQ 882

877 R.............KVGRNDPCPCGSGKKYKQCHGRLQ 902
    I             :..II.IIIIIIIII:IIII.I
883 REALPVSALPEGWDRTNRNAPCPCGSGKKFKQCHGSLVR 921
```

FIG. 3. Comparison of the predicted amino acid sequence of the SecA protein from E. coli (top line) and C. crescentus (bottom line), using the GAP program from the Genetics Computer Group package (4). Vertical lines indicate identical amino acid residues, and dots indicate similar amino acid residues.

30°C 37°C   30°C 37°C
WT   secA⁻

FIG. 4. Accumulation of the precursor protein of β-lactamase in the C. crescentus secA mutant strain LS416. Wild-type (WT) and secA mutant cells carrying an RP4-derived plasmid encoding β-lactamase were labeled with [³⁵S]methionine for 5 min at 30 or 37°C after 5 min of preincubation at the same temperature. Equivalent amounts of cell lysates were immunoprecipitated with anti-β-lactamase antibody and were analyzed by SDS-7.5% polyacrylamide gel electrophoresis followed by fluorography. m, mature-size protein; p, precursor.

visible polar structures. These observations suggest that strain LS416 is defective in both stalk formation and cell division at the nonpermissive temperature.

Because the secA mutant is unable to form a stalk at the nonpermissive temperature, we tested other events that normally occur during the swarmer-to-stalked cell transition. These include the turnover of the chemotaxis proteins (2, 14) and the initiation of DNA replication (3, 5, 20). The steady-state levels of the chemoreceptors were examined in synchronized cultures of strain LS416 grown at 30 and 37°C by immunoblot analysis using anti-McpA antiserum (Fig. 6). The turnover of the McpA chemoreceptor and its reappearance in the predivisional cell due to its de novo synthesis (1, 2)
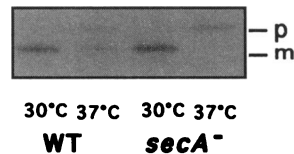
occurred normally at 30°C. The McpA chemoreceptors present in swarmer cells of the secA mutant grown at 37°C were degraded at the normal time in the cell cycle but failed to reappear at later times. These results indicate that the temporally controlled proteolysis of McpA does not require SecA function. The observation that at 37°C McpA did not reappear suggests that the arrest in the cell cycle occurred prior to its de novo synthesis.

The chemotaxis and flagellar proteins normally initiate synthesis at a defined time in the cell cycle, prior to cell division (2, 13, 14, 36). To more precisely define the cell cycle arrest of the secA mutant strain, the de novo synthesis of chemoreceptors and flagellins was examined in synchronized cultures at both the permissive and nonpermissive temperatures (Fig. 7). Swarmer cells were isolated from wild-type and mutant cultures grown at 30°C. In each case, half of the culture was shifted to 37°C and the rest was allowed to continue growth at 30°C. Aliquots of pulse-labeled cells were immunoprecipitated with antibodies to the McpA chemoreceptors and to flagellins. At the permissive temperature, the de novo synthesis of McpA and the flagellins initiated midway through the cell cycle both in wild-type cells and in the secA mutant cells (Fig. 7). At the nonpermissive temperature, however, McpA and the flagellins were not synthesized in the secA mutant cells but were synthesized in the wild-type control. These results suggest that either the cell cycle of the secA mutant culture was arrested prior to the time of de novo McpA and flagellin synthesis or chemoreceptors and flagellins were synthesized but rapidly degraded because of their improper localization at the non-permissive temperature.

The initiation of DNA replication is independent of SecA function in the LS416 mutant strain. To assay the initiation of DNA replication at the nonpermissive temperature in synchronized culture of the secA LS416 mutant strain, cultures were examined by FACS analysis. This technique allows measurement of the DNA content of individual cells (as intensity of fluorescence of DNA-associated chromomycin A3) with a large sample size. Swarmer cells of strain LS416 grown at 30°C were isolated and shifted to the nonpermissive temperature. Chromosome replication was monitored in synchronously growing cultures by FACS analysis after the cells were treated with chromomycin A3 (Fig. 8). Cultures incubated at 30°C for 150 min exhibited two peaks, representing cells with one chromosome (swarmer cells) and two chromosomes (predivisional cells). In contrast, synchronized cultures of LS416 incubated at the nonpermissive temperature showed only one peak which was positioned between the one- and two-chromosome peaks. These results (shown diagrammatically in Fig. 9) suggest that the C. crescentus secA mutant cells are able to initiate DNA replication at the nonpermissive temperature but
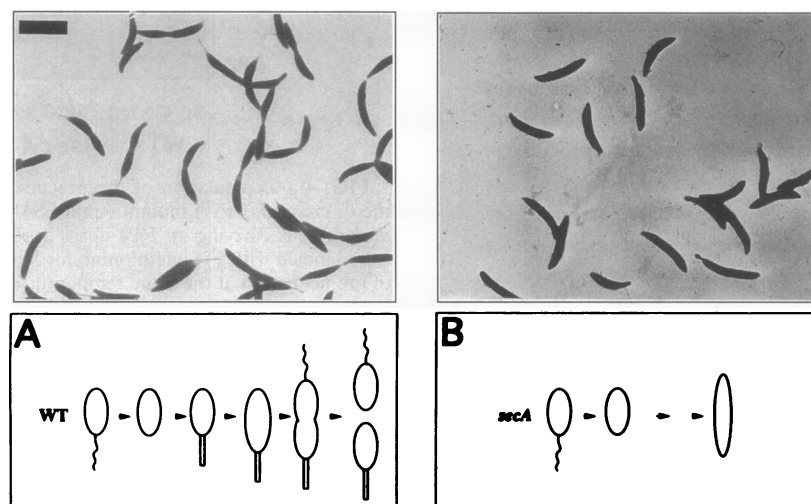
FIG. 5. Morphology of the *C. crescentus* *secA* mutant at 37°C. Swarmer cells isolated from cultures of the wild-type (WT) parent (LS107) or the *secA* mutant strain (LS416) grown in PYE medium at 30°C were incubated at 37°C for 150 min and then examined by electron microscopy. Schematic diagrams of the observed cell cycle progression of LS107 (A) and the *secA* mutant strain LS416 (B) at 37°C are shown below the respective electron micrographs. The size bar represents 2 μm, and the two micrographs are the same magnification.

exhibit a cell cycle arrest before the completion of chromosome replication.

DISCUSSION

A group of ts mutants of *C. crescentus* that arrest at specific stages of the cell cycle have been isolated. The phenotype of one of these mutants, which arrests with cells lacking visible polar structures, was corrected by a clone containing the homolog of the *E. coli* *secA* gene. DNA sequence analysis of the cloned gene revealed that the predicted amino acid sequence has 53% identity to the *E. coli* SecA protein sequence along its entire length.

E. coli *secA* mutants were initially isolated from genetic screens for mutants defective in protein secretion (27). Biochemical characterization of the *E. coli* SecA protein showed the direct involvement of SecA in protein translocation (26). In

FIG. 6. Turnover of McpA chemoreceptor in the *secA* mutant strain. Swarmer cells isolated from cultures of *secA* mutant strain LS416 grown in PYE medium at 30°C were allowed to proceed through the cell cycle at 30 or 37°C. At the indicated time points, cell lysates were prepared and used for immunoblot analysis with anti-McpA antiserum as described in Materials and Methods. A cell cycle diagram is shown above the immunoblot.

Bacillus subtilis, a *secA* mutant was initially isolated as a *div* mutant because it was defective in cell division (32). Subsequent cloning and sequencing of this *div* gene revealed that it was a *B. subtilis* homolog of the *E. coli* *secA* gene (33).

Since the *C. crescentus* *secA* mutant appeared to arrest at the nonpermissive temperature as elongated cells with no visible polar structures, we examined synchronized cultures to determine whether they were able to pass the swarmer-to-stalked cell transition point. During this transition, the flagellum, pili, DNA phage receptors, and chemoreceptors are lost (2, 11, 14, 36) and stalk formation is initiated at the polar site previously occupied by the flagellum (35). DNA replication is initiated as the cells enter the stalked cell stage (3, 20). Although stalks failed to form after the release of the flagellum in the *secA* mutant, both the turnover of the chemoreceptors and the initiation of DNA replication occurred at the nonpermissive temperature, suggesting that these cells had passed the swarmer-to-stalked cell transition point before the cell cycle block. Assays of the de novo synthesis of the chemoreceptors and the flagellins revealed that the cell cycle arrest occurred prior to their normal stage of synthesis. A schematic diagram of the cell cycle analysis of *secA* mutant strain LS416 at 30 and 37°C, shown in Fig. 9, proposes that the cell cycle arrest of the *secA* mutant occurs after the swarmer-to-stalked transition but prior to the predivisional cell stage.

It was previously shown that *C. crescentus* mutants auxotrophic for fatty acids have defects in DNA replication (15, 16), suggesting that interactions between the chromosome and the cytoplasmic membrane may be important for the initiation of replication. SecA-dependent events, however, do not appear to mediate this interaction because the *secA* mutant allele in strain LS416 initiated DNA replication at the correct time in the cell cycle at the nonpermissive temperature.

It is possible to interpret the observed cell cycle arrest phenotype of the *secA* mutant as a result of swarmer cell death upon a shift to the nonpermissive temperature, prior to differentiation into predivisional cells. This is unlikely, however, because the initial mutant screen was for bacteriostatic ts mutants by using the cephalosporin C enrichment process, in which mutagenized cells were incubated at 37°C for 14 h
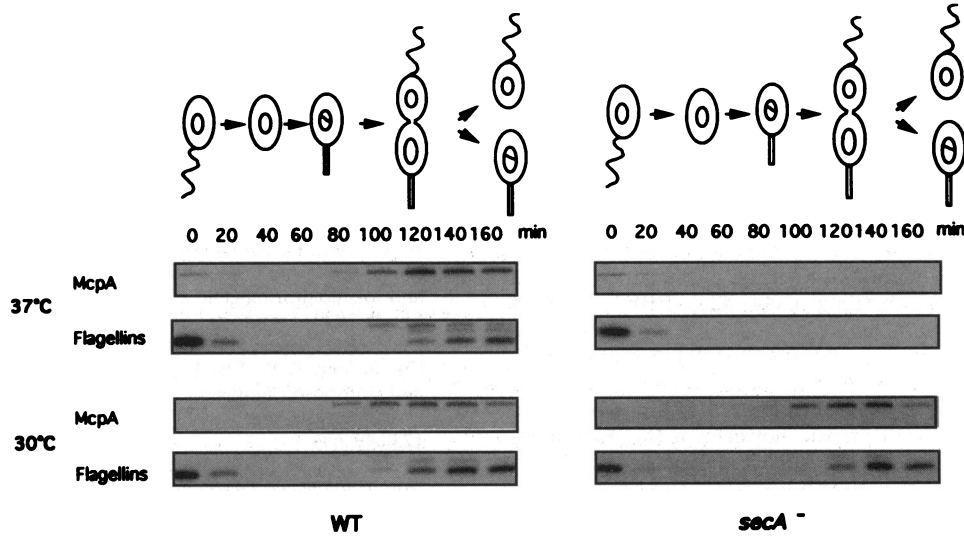
FIG. 7. Synthesis of McpA and flagellins during the *C. crescentus* cell cycle in the wild-type (WT) parent LS107 and *secA* mutant LS416 strains. Swarmer cells isolated from cultures of both strains grown in M2G medium at 30°C were allowed to proceed through the cell cycle at 30 or 37°C. At 20-min intervals, synchronous cells were pulse-labeled with [^{35}S]methionine for 5 min and harvested as described in Materials and Methods. The syntheses of McpA and flagellins were assayed by immunoprecipitation with anti-McpA and antiflagellin antisera, respectively. The three flagellins are 25, 27.5, and 29 kDa. A cell cycle diagram is shown above each autoradiogram.

before selection of ts mutants. The *C. crescentus secA* mutant remained viable for several generations after being shifted to the nonpermissive temperature, as was shown to be the case for *E. coli secA* mutants (27). We do not know the mechanism whereby cells with a partially replicated chromosome remain viable and resume growth and replication upon shift-down to the permissive temperature. Clearly, *secA* mutations cause pleiotropic effects (27, 32), and the observed bacteriostatic DNA elongation phenotype may be indirect.

Stalk formation and cell division appear to require a functional SecA protein. The stalk is a slender polar rod whose membrane and cell wall are continuous with those of the cell body and whose cytoplasm is devoid of DNA and ribosomes (18, 31). The stalk is formed at the cell pole by an annular ring of cell wall and membrane synthesis, in many ways analogous to a polar cell division event. Growth of the *C. crescentus secA* mutant at the restrictive temperature blocks both cell division and the formation of the stalk. Indeed, cell division is commonly defective in bacteria with mutant *secA* alleles (27, 32, 33).

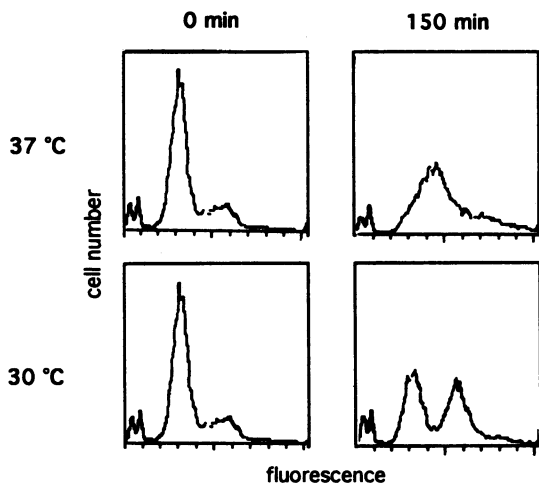Although several stalkless *C. crescentus* mutants have been

FIG. 8. FACS analysis of chromosome replication in the *secA* mutant strain LS416. Swarmer cells of the *secA* mutant LS416 were isolated from cultures grown in PYE medium at 30°C and allowed to proceed through the cell cycle at 30 or 37°C. At 0 and 150 min, cells were fixed with 70% ethanol. The chromosome replication state was monitored by FACS after staining the fixed cells with chromomycin A3. The ordinate indicates the relative number of cells, and abscissa indicates the fluorescence intensity (DNA content) of single cells in arbitrary units on a linear scale.
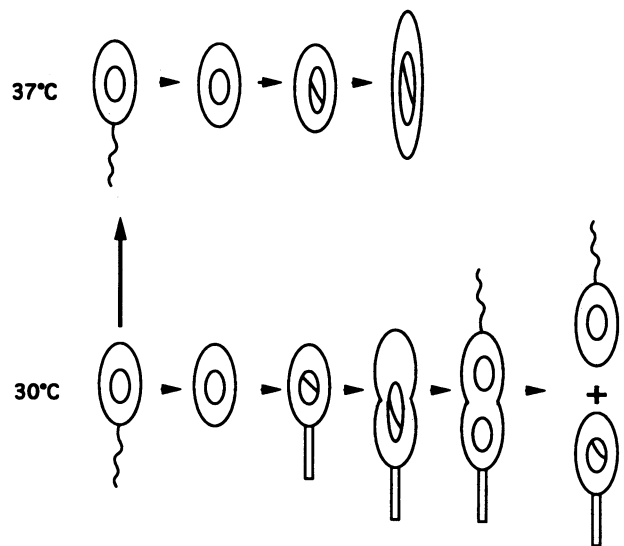

FIG. 9. Schematic of the observed cell cycle arrest of the *C. crescentus secA* mutant shifted to the restrictive temperature at the swarmer cell stage (upward-pointing arrow).

reported (10, 21, 28), the biosynthesis and regulation of stalk formation are not well defined. The apparent requirement for SecA function for stalk biogenesis revealed in this study suggests that proteins necessary for stalk formation require SecA-mediated protein translocation. Candidate proteins include the penicillin-binding proteins (PBPs). In *E. coli*, seven different PBPs have been identified (for a review, see reference 43). These proteins exhibit transpeptidase or transglycosylase activity involved in *E. coli* cell wall biosynthesis and are localized to the cytoplasmic membrane. Several *C. crescentus* PBPs were previously identified by using radioactively labeled penicillin derivatives in binding assays (23). PBP2, which is encoded by the *pbpA* gene in *E. coli* (40), appears to be required for stalk biosynthesis because a stalkless mutant of *C. crescentus*, initially isolated as a ts cell cycle arrest mutant, was shown to have a mutation in the *C. crescentus* homolog of the *pbpA* gene (19a). It is not known, however, if the membrane localization of the *E. coli* PBP2 is SecA dependent.

SecA is required for flagellum biogenesis. The period of the transition of the stalked cell to the predivisional cell is a time of de novo biogenesis of the flagellum. We speculated above that proper translocation of proteins is necessary for stalk formation. In the same sense, blocking the proper translocation of proteins may inhibit the assembly of the flagellum. We have shown that the *C. crescentus secA* mutant strain does not synthesize flagellins at the nonpermissive temperature. Since flagellins are at the lowest level of the hierarchy of flagellar gene expression (for reviews, see references 13 and 24), it is possible that blocking protein secretion or protein insertion into the membrane leads to defects in flagellar assembly that, in turn, inhibit expression of the genes at other levels of the hierarchy. Alternatively, the block in flagellum biogenesis observed in the *secA* mutant strain at the nonpermissive temperature may be an indirect consequence of arresting the cell cycle prior to the signal that initiates the flagellar transcriptional hierarchy. It is also possible that incomplete DNA replication observed in the *secA* mutant at the nonpermissive temperature inhibits flagellar gene expression, since flagellar gene transcription is prevented by a disruption in DNA synthesis (39).

In summary, we have described the cell cycle arrest phenotype of a *C. crescentus secA* mutant and proposed that proper translocation of proteins is important for polar morphogenesis and cell division but not for the initiation of DNA replication. Although the cell cycle arrest phenotype of the *C. crescentus secA* mutant is likely to be an indirect effect of protein translocation defects, this mutation in the *secA* gene allows the identification of morphogenetic events that require SecA-dependent protein translocation.

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